

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
9 October 2003 (09.10.2003)

PCT

(10) International Publication Number
WO 03/083125 A1

(51) International Patent Classification⁷: C12P 21/06, C12N 9/54, 1/20, 15/00, 15/74, 1/00

(74) Agent: MACKNIGHT, Kamrin, T.; GENENCOR INTERNATIONAL, INC., 925 Page Mill Road, Palo Alto, CA 94304 (US).

(21) International Application Number: PCT/US03/09585

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date: 28 March 2003 (28.03.2003)

(25) Filing Language: English

(26) Publication Language: English

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(30) Priority Data:
60/368,949 29 March 2002 (29.03.2002) US
60/368,858 29 March 2002 (29.03.2002) US
60/376,343 29 April 2002 (29.04.2002) US

(71) Applicant (*for all designated States except US*): GENENCOR INTERNATIONAL, INC. [US/US]; 925 Page Mill Road, Palo Alto, CA 94304 (US).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 03/083125 A1

(54) Title: EHANCED PROTEIN EXPRESSION IN *BACILLUS*

(57) Abstract: The present invention provides cells that have been genetically manipulated to have an altered capacity to produce expressed proteins. In particular, the present invention relates to Gram-positive microorganisms, such as *Bacillus* species having enhanced expression of a protein of interest, wherein one or more chromosomal genes have been inactivated, and preferably wherein one or more chromosomal genes have been deleted from the *Bacillus* chromosome. In some further embodiments, one or more indigenous chromosomal regions have been deleted from a corresponding wild-type *Bacillus* host chromosome.

ENHANCED PROTEIN EXPRESSION IN *BACILLUS*

5

FIELD OF THE INVENTION

The present invention provides cells that have been genetically manipulated to have an altered capacity to produce expressed proteins. In particular, the present 10 invention relates to Gram-positive microorganisms, such as *Bacillus* species having enhanced expression of a protein of interest, wherein one or more chromosomal genes have been inactivated, and preferably wherein one or more chromosomal genes have been deleted from the *Bacillus* chromosome. In some further embodiments, one or more indigenous chromosomal regions have been deleted from a corresponding wild-type 15 *Bacillus* host chromosome.

BACKGROUND OF THE INVENTION

Genetic engineering has allowed the improvement of microorganisms used as industrial bioreactors, cell factories and in food fermentations. In particular, *Bacillus* 20 species produce and secrete a large number of useful proteins and metabolites (Zukowski, "Production of commercially valuable products," *In: Doi and McGloaglin (eds.) Biology of Bacilli: Applications to Industry*, Butterworth-Heinemann, Stoneham. Mass pp 311-337 [1992]). The most common *Bacillus* species used in industry are *B. licheniformis*, *B. amyloliquefaciens* and *B. subtilis*. Because of their GRAS (generally recognized as 25 safe) status, strains of these *Bacillus* species are natural candidates for the production of proteins utilized in the food and pharmaceutical industries. Important production enzymes include α -amylases, neutral proteases, and alkaline (or serine) proteases. However, in spite of advances in the understanding of production of proteins in *Bacillus* host cells, there remains a need for methods to increase expression of these proteins.

30

SUMMARY OF THE INVENTION

The present invention provides cells that have been genetically manipulated to have an altered capacity to produce expressed proteins. In particular, the present 35 invention relates to Gram-positive microorganisms, such as *Bacillus* species having enhanced expression of a protein of interest, wherein one or more chromosomal genes have been inactivated, and preferably wherein one or more chromosomal genes have

- 2 -

been deleted from the *Bacillus* chromosome. In some further embodiments, one or more indigenous chromosomal regions have been deleted from a corresponding wild-type *Bacillus* host chromosome. In some preferred embodiments, the present invention provides methods and compositions for the improved expression and/or secretion of a protein of interest in *Bacillus*.

In particularly preferred embodiments, the present invention provides means for improved expression and/or secretion of a protein of interest in *Bacillus*. More particularly, in these embodiments, the present invention involves inactivation of one or more chromosomal genes in a *Bacillus* host strain, wherein the inactivated genes are not necessary for strain viability. One result of inactivating one or more of the chromosomal genes is the production of an altered *Bacillus* strain that is able to express a higher level of a protein of interest over a corresponding non-altered *Bacillus* host strain.

Furthermore, in alternative embodiments, the present invention provides means for removing large regions of chromosomal DNA in a *Bacillus* host strain, wherein the deleted indigenous chromosomal region is not necessary for strain viability. One result of removing one or more indigenous chromosomal regions is the production of an altered *Bacillus* strain that is able to express a higher level of a protein of interest over a corresponding unaltered *Bacillus* strain. In some preferred embodiments, the *Bacillus* host strain is a recombinant host strain comprising a polynucleotide encoding a protein of interest. In some particularly preferred embodiments, the altered *Bacillus* strain is a *B. subtilis* strain. As explained in detail below, deleted indigenous chromosomal regions include, but are not limited to prophage regions, antimicrobial (e.g., antibiotic) regions, regulator regions, multi-contiguous single gene regions and operon regions.

In some embodiments, the present invention provides methods and compositions for enhancing expression of a protein of interest from a *Bacillus* cell. In some preferred embodiments, the methods comprise inactivating one or more chromosomal genes selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spollSA*, *sigB*, *phrC*, *rapA*, *CssS trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD* in a *Bacillus* host strain to produce an altered *Bacillus* strain; growing the altered *Bacillus* strain under suitable growth conditions; and allowing a protein of interest to be expressed in the altered *Bacillus*, wherein the expression of the protein is enhanced, compared to the corresponding unaltered *Bacillus* host strain. In some embodiments, the protein of interest is a homologous protein, while in other embodiments, the protein of interest is a heterologous protein. In some embodiments, more than one protein of interest is produced. In some preferred embodiments, the *Bacillus* species is a *B. subtilis* strain. In yet

- 3 -

further embodiments, inactivation of a chromosomal gene comprises the deletion of a gene to produce the altered *Bacillus* strain. In additional embodiments, inactivation of a chromosomal gene comprises insertional inactivation. In some preferred embodiments, the protein of interest is an enzyme. In some embodiments, the protein of interest is selected from proteases, cellulases, amylases, carbohydrases, lipases, isomerases, transferases, kinases and phosphatases, while in other embodiments, the protein of interest is selected from the group consisting of antibodies, hormones and growth factors.

In yet additional embodiments, the present invention provides altered *Bacillus* strains comprising the deletion of one or more chromosomal genes selected from the group of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*. In some embodiments, the altered strain is a protease producing *Bacillus* strain. In an alternative embodiment, the altered *Bacillus* strain is a subtilisin producing strain. In yet other embodiments, the altered *Bacillus* strain further comprises a mutation in a gene selected from the group consisting of *degU*, *degQ*, *degS*, *scoC4*, *spolIE*, and *oppA*.

In further embodiments, the present invention provides DNA constructs comprising an incoming sequence. In some embodiments, the incoming sequence includes a selective marker and a gene or gene fragment selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*. In alternative embodiments, the selective marker is located in between two fragments of the gene. In other embodiments, the incoming sequence comprises a selective marker and a homology box, wherein the homology box flanks the 5' and/or 3' end of the marker. In additional embodiments, a host cell is transformed with the DNA construct. In further embodiments, the host cell is an *E. coli* or a *Bacillus* cell. In some preferred embodiments, the DNA construct is chromosomally integrated into the host cell.

The present invention also provides methods for obtaining an altered *Bacillus* strain expressing a protein of interest which comprises transforming a *Bacillus* host cell with the DNA construct of the present, wherein the DNA construct is integrated into the chromosome of the *Bacillus* host cell; producing an altered *Bacillus* strain, wherein one or more chromosomal genes have been inactivated; and growing the altered *Bacillus* strain under suitable growth conditions for the expression of a protein of interest. In some embodiments, the protein of interest is selected from proteases, cellulases, amylases, carbohydrases, lipases, isomerases, transferases, kinases and phosphatases, while in other embodiments, the protein of interest is selected from the group consisting of antibodies, hormones and

- 4 -

growth factors. In yet additional embodiments, the *Bacillus* host strain is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. thuringiensis*, *B. clausii*, *B. megaterium*, and preferably, *B. subtilis*. In some embodiments, the *Bacillus* host strain is a recombinant host. In yet additional embodiments, the protein of interest is recovered. In further embodiments, the selective marker is excised from the altered *Bacillus*.

The present invention further provides methods for obtaining an altered *Bacillus* strain expressing a protein of interest. In some embodiments, the method comprises transforming a *Bacillus* host cell with a DNA construct comprising an incoming sequence wherein the incoming sequence comprises a selective marker and a gene selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spollSA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*, wherein the DNA construct is integrated into the chromosome of the *Bacillus* host cell and results in the deletion of one or more gene(s); obtaining an altered *Bacillus* strain, and growing the altered *Bacillus* strain under suitable growth conditions for the expression of the protein of interest.

In some alternative embodiments, the present invention provides a DNA construct comprising an incoming sequence, wherein the incoming sequence includes a selective marker and a *cssS* gene, a *cssS* gene fragment or a homologous sequence thereto. In some embodiments, the selective marker is located between two fragments of the gene. In alternative embodiments, the incoming sequence comprises a selective marker and a homology box wherein the homology box flanks the 5' and/or 3' end of the marker. In yet other embodiments, a host cell is transformed with the DNA construct. In additional embodiments, the host cell is an *E. coli* or a *Bacillus* cell. In still further embodiments, the DNA construct is chromosomally integrated into the host cell.

The present invention also provides methods for obtaining *Bacillus subtilis* strains that demonstrate enhanced protease production. In some embodiments, the methods comprise the steps of transforming a *Bacillus subtilis* host cell with a DNA construct according to the invention; allowing homologous recombination of the DNA construct and a homologous region of the *Bacillus* chromosome wherein at least one of the following genes, *sbo*, *slr*, *ybcO*, *csn*, *spollSA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*, is deleted from the *Bacillus* chromosome; obtaining an altered *Bacillus subtilis* strain; and growing the altered *Bacillus* strain under conditions suitable for the expression of a protease. In some embodiments, the protease producing *Bacillus* is a subtilisin producing strain. In alternative embodiments, the

- 5 -

protease is a heterologous protease. In additional embodiments, the protease producing strain further includes a mutation in a gene selected from the group consisting of *degU*, *degQ*, *degS*, *scoC4*, *spolIIE*, and *oppA*. In some embodiments, the inactivation comprises the insertional inactivation of the gene.

5 The present invention further provides altered *Bacillus subtilis* strains comprising a deletion of one or more chromosomal genes selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*, wherein the altered *Bacillus subtilis* strain is capable of expressing a protein of interest. In some
10 embodiments, the protein of interest is an enzyme. In some additional embodiments, the protein of interest is a heterologous protein.

In some embodiments, the present invention provides altered *Bacillus* strains comprising a deletion of one or more indigenous chromosomal regions or fragments thereof, wherein the indigenous chromosomal region includes about 0.5 to 500 kilobases
15 (kb) and wherein the altered *Bacillus* strains have an enhanced level of expression of a protein of interest compared to the corresponding unaltered *Bacillus* strains when grown under essentially the same growth conditions.

In yet additional embodiments, the present invention provides protease-producing *Bacillus* strains which comprise at least one deletion of an indigenous chromosomal region
20 selected from the group consisting of a PBSX region, a skin region, a prophage 7 region, a SP β region, a prophage 1 region, a prophage 2 region, a prophage 3 region, a prophage 4 region, a prophage 5 region, a prophage 6 region, a PPS region, a PKS region, a *yvF-yvK* region, a DHB region and fragments thereof.

In further embodiments, the present invention provides methods for enhancing the expression of a protein of interest in *Bacillus* comprising: obtaining an altered *Bacillus* strain
25 produced by introducing a DNA construct including a selective marker and an inactivating chromosomal segment into a *Bacillus* host strain, wherein the DNA construct is integrated into the *Bacillus* chromosome resulting in the deletion of an indigenous chromosomal region or fragment thereof from the *Bacillus* host cell; and growing the altered *Bacillus* strain under
30 suitable growth conditions, wherein expression of a protein of interest is greater in the altered *Bacillus* strain compared to the expression of the protein of interest in the corresponding unaltered *Bacillus* host cell.

The present invention also provides methods for obtaining a protein of interest from a *Bacillus* strain comprising the steps of: transforming a *Bacillus* host cell with a DNA construct
35 which comprises a selective marker and an inactivating chromosomal segment, wherein the

- 6 -

DNA construct is integrated into the chromosome of the *Bacillus* strain and results in deletion of an indigenous chromosomal region or fragment thereof to form an altered *Bacillus* strain; culturing the altered *Bacillus* strain under suitable growth conditions to allow the expression of a protein of interest; and recovering the protein of interest.

- 5 The present invention also provides a means for the use of DNA microarray data to screen and/or identify beneficial mutations. In some particularly preferred embodiments, these mutations involve genes selected from the group consisting of *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*. In some preferred embodiments, these beneficial mutations are based on transcriptome evidence for the simultaneous expression of a given amino acid biosynthetic pathway and biodegradative pathway, and/or evidence that deletion of the degradative pathway results in a better performing strain and/or evidence that overexpression of the biosynthetic pathway results in a better performing strain. In additional embodiments, the present invention provides means for the use of DNA microarray data to provide beneficial mutations. In some particularly preferred 10 embodiments, these mutations involve genes selected from the group consisting of *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*, when the expression of mRNA from genes comprising an amino acid biosynthetic pathway is not balanced and overexpression of the entire pathway provides a better performing strain than the parent (i.e., wild-type and/or originating) strain. Furthermore, the present invention provides means to 15 improve production strains through the inactivation of gluconeogenic genes. In some of these preferred embodiments, the inactivated gluconeogenic genes are selected from the group consisting of *pckA*, *gapB*, and *fbp*.

- The present invention provides methods for enhancing expression of a protein of interest from *Bacillus* comprising the steps of obtaining an altered *Bacillus* strain capable of 20 producing a protein of interest, wherein the altered *Bacillus* strain has at least one inactivated chromosomal gene selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*, and growing the altered *Bacillus* strain under 25 conditions such that the protein of interest is expressed by the altered *Bacillus* strain, wherein the expression of the protein of interest is enhanced, compared to the expression of the protein of interest in an unaltered *Bacillus* host strain. In some embodiments, the protein of interest is selected from the group consisting of homologous proteins and heterologous proteins. In some embodiments, the protein of interest is selected from proteases, cellulases, amylases, carbohydrases, lipases, isomerases, transferases, kinases and phosphatases, 30 while in other embodiments, the protein of interest is selected from the group consisting of

- 7 -

antibodies, hormones and growth factors. In some particularly preferred embodiments, the protein of interest is a protease. In additional embodiments, the altered *Bacillus* strain is obtained by deleting one or more chromosomal genes selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*.

The present invention also provides altered *Bacillus* strains obtained using the method described herein. In some preferred embodiments, the altered *Bacillus* strains comprise a chromosomal deletion of one or more genes selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*. In some embodiments, more than one of these chromosomal genes have been deleted. In some particularly preferred embodiments, the altered strains are *B. subtilis* strains. In additional preferred embodiments, the altered *Bacillus* strains are protease producing strains. In some particularly preferred embodiments, the protease is a subtilisin. In yet additional embodiments, the subtilisin is selected from the group consisting of subtilisin 168, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin 147, subtilisin 309 and variants thereof. In yet further embodiments, altered *Bacillus* strains further comprise mutation(s) in at least one gene selected from the group consisting of *degU*, *degQ*, *degS*, *scoC4*, *spolIE*, and *oppA*. In some particularly preferred embodiments, the altered *Bacillus* strains further comprise a heterologous protein of interest.

The present invention also provides DNA constructs comprising at least one gene selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*, gene fragments thereof, and homologous sequences thereto. In some preferred embodiments, the DNA constructs comprise at least one nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 37, SEQ ID NO: 25, SEQ ID NO: 21, SEQ ID NO: 50, SEQ ID NO: 29, SEQ ID NO: 23, SEQ ID NO: 27, SEQ ID NO: 19, SEQ ID NO: 31, SEQ ID NO: 48, SEQ ID NO: 46, SEQ ID NO: 35, and SEQ ID NO: 33. In some embodiments, the DNA constructs further comprise at least one polynucleotide sequence encoding at least one protein of interest.

The present invention also provides plasmids comprising the DNA constructs. In further embodiments, the present invention provides host cells comprising the plasmids

- 8 -

comprising the DNA constructs. In some embodiments, the host cells are selected from the group consisting of *Bacillus* cells and *E. coli* cells. In some preferred embodiments, the host cell is *B. subtilis*. In some particularly preferred embodiments, the DNA construct is integrated into the chromosome of the host cell. In alternative embodiments, the DNA
5 construct comprises at least one gene that encodes at least one amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 38, SEQ ID NO: 26, SEQ ID NO: 22, SEQ ID NO: 57, SEQ ID NO: 30, SEQ
10 ID NO: 24, SEQ ID NO: 28, SEQ ID NO: 20, SEQ ID NO: 32, SEQ ID NO: 55, SEQ ID NO: 53, SEQ ID NO: 36, and SEQ ID NO: 34. In additional embodiments, the DNA constructs further comprise at least one selective marker, wherein the selective marker is flanked on each side by a fragment of the gene or homologous gene sequence thereto.

The present invention also provides DNA constructs comprising an incoming
15 sequence, wherein the incoming sequence comprises a nucleic acid encoding a protein of interest, and a selective marker flanked on each side with a homology box, wherein the homology box includes nucleic acid sequences having 80 to 100% sequence identity to the sequence immediately flanking the coding regions of at least one gene selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*,
20 *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*. In some embodiments, the DNA constructs further comprise at least one nucleic acids which flanks the coding sequence of the gene. The present invention also provides plasmids comprising the DNA constructs. In further embodiments, the present invention provides host cells comprising the plasmids comprising the DNA constructs. In some embodiments, the host cells are selected from the group consisting of *Bacillus* cells and *E. coli* cells. In some preferred embodiments, the host cell is *B. subtilis*. In some particularly preferred
25 embodiments, the DNA construct is integrated into the chromosome of the host cell. In additional preferred embodiments, the selective marker has been excised from the host cell chromosome.

The present invention further provides methods for obtaining an altered *Bacillus* strain
30 with enhanced protease production comprising: transforming a *Bacillus* host cell with at least one DNA construct of the present invention, wherein the protein of interest in the DNA construct is a protease, and wherein the DNA construct is integrated into the chromosome of the *Bacillus* host cell under conditions such that at least one gene is inactivated to produce
35 an altered *Bacillus* strain; and growing the altered *Bacillus* strain under conditions such that

- 9 -

enhanced protease production is obtained. In some particularly preferred embodiments, the method further comprises recovering the protease. In alternative preferred embodiments, at least one inactivated gene is deleted from the chromosome of the altered *Bacillus* strain. The present invention also provides altered *Bacillus* strains produced using the methods described herein. In some embodiments, the *Bacillus* host strain is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. laetus*, *B. clausii*, *B. megaterium*, and *B. thuringiensis*. In some preferred embodiments, the *Bacillus* host cell is *B. subtilis*.

The present invention also provides methods for enhancing expression of a protease in an altered *Bacillus* comprising: transforming a *Bacillus* host cell with a DNA construct of the present invention; allowing homologous recombination of the DNA construct and a region of the chromosome of the *Bacillus* host cell, wherein at least one gene of the chromosome of the *Bacillus* host cell is inactivated, to produce an altered *Bacillus* strain; and growing the altered *Bacillus* strain under conditions suitable for the expression of the protease, wherein the production of the protease is greater in the altered *Bacillus subtilis* strain compared to the *Bacillus subtilis* host prior to transformation. In some preferred embodiments, the protease is subtilisin. In additional embodiments, the protease is a recombinant protease. In yet further embodiments, inactivation is achieved by deletion of at least one gene. In still further embodiments, inactivation is by insertional inactivation of at least one gene. The present invention also provides altered *Bacillus* strains obtained using the methods described herein. In some embodiments, altered *Bacillus* strain comprises at least one inactivated gene selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spoII SA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*. In some preferred embodiments, the inactivated gene has been inactivated by deletion. In additional embodiments, the altered *Bacillus* strains further comprise at least one mutation in a gene selected from the group consisting of *degU*, *degS*, *degQ*, *scoC4*, *spoII E*, and *oppA*. In some preferred embodiments, the mutation is *degU(Hy)32*. In still further embodiments, the strain is a recombinant protease producing strain. In some preferred embodiments, the altered *Bacillus* strains are selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. laetus*, *B. clausii*, *B. megaterium*, and *B. thuringiensis*.

The present invention also provides altered *Bacillus* strains comprising a deletion of one or more indigenous chromosomal regions or fragments thereof, wherein the Indigenous

- 10 -

chromosomal region includes about 0.5 to 500 kb, and wherein the altered *Bacillus* strain has an enhanced level of expression of a protein of interest compared to a corresponding unaltered *Bacillus* strain when the altered and unaltered *Bacillus* strains are grown under essentially the same growth conditions. In preferred embodiments, the altered *Bacillus* strain is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. lautus*, *B. clausii*, *B. megaterium*, and *B. thuringiensis*. In some preferred embodiments, the altered *Bacillus* strain is selected from the group consisting of *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*. In some particularly preferred embodiments, the altered *Bacillus* strain is a *B. subtilis* strain. In yet further embodiments, the indigenous chromosomal region is selected from the group consisting of a PBSX region, a skin region, a prophage 7 region, a SP β region, a prophage 1 region, a prophage 2 region, a prophage 4 region, a prophage 3 region, a prophage 4 region, a prophage 5 region, a prophage 6 region, a PPS region, a PKS region, a YVFF-YVEK region, a DHB region and fragments thereof. In some preferred embodiments, two indigenous chromosomal regions or fragments thereof have been deleted. In some embodiments, the protein of interest is selected from proteases, cellulases, amylases, carbohydrases, lipases, isomerases, transferases, kinases and phosphatases, while in other embodiments, the protein of interest is selected from the group consisting of antibodies, hormones and growth factors. In yet additional embodiments, the protein of interest is a protease. In some preferred embodiments, the protease is a subtilisin. In some particularly preferred embodiments, the subtilisin is selected from the group consisting of subtilisin 168, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin 147 and subtilisin 309 and variants thereof. In further preferred embodiments, the *Bacillus* host is a recombinant strain. In some particularly preferred embodiments, the altered *Bacillus* strains further comprise at least one mutation in a gene selected from the group consisting of *degU*, *degQ*, *degS*, *sco4*, *spolIE* and *oppA*. In some preferred embodiments, the mutation is *degU(Hy)32*.

The present invention further provides protease producing *Bacillus* strains comprising a deletion of an indigenous chromosomal region selected from the group consisting of a PBSX region, a skin region, a prophage 7 region, a SP β region, a prophage 1 region, a prophage 2 region, a prophage 3 region, a prophage 4 region, a prophage 5 region, a prophage 6 region, a PPS region, a PKS region, a YVFF-YVEK region, a DHB region and fragments thereof. In some preferred embodiments, the protease is a subtilisin. In some embodiments, the protease is a heterologous protease. In some preferred embodiments, the altered *Bacillus* strain is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B.*

- 11 -

subtilis, *B. amyloliquefaciens* *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. laetus*, *B. clausii*, *B. megaterium*, and *B. thuringiensis*. In additional embodiments, the *Bacillus* strain is a *B. subtilis* strain.

The present invention also provides methods for enhancing the expression of a protein of interest in *Bacillus* comprising: introducing a DNA construct including a selective marker and an inactivating chromosomal segment into a *Bacillus* host strain, wherein the DNA construct is integrated into the chromosome of the *Bacillus* host strain, resulting in the deletion of an indigenous chromosomal region or fragment thereof from the *Bacillus* host cell to produce an altered *Bacillus* strain; and growing the altered *Bacillus* strain under suitable conditions, wherein expression of a protein of interest is greater in the altered *Bacillus* strain compared to the expression of the protein of interest in a *Bacillus* host cell that has not been altered. In some preferred embodiments, the methods further comprise the step of recovering the protein of interest. In some embodiments, the methods further comprise the step of excising the selective marker from the altered *Bacillus* strain. In additional embodiments, the indigenous chromosomal region is selected from the group of regions consisting of PBSX, skin, prophage 7, SP β , prophage 1, prophage 2, prophage 3, prophage 4, prophage 5, prophage 6, PPS, PKS, YVFF-YVEK, DHB and fragments thereof. In further embodiments, the altered *Bacillus* strain comprises deletion of at least two indigenous chromosomal regions. In some preferred embodiments, the protein of interest is an enzyme. In some embodiments, the protein of interest is selected from proteases, cellulases, amylases, carbohydrases, lipases, isomerases, transferases, kinases and phosphatases, while in other embodiments, the protein of interest is selected from the group consisting of antibodies, hormones and growth factors. In some embodiments, the *Bacillus* host strain is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens* *B. brevis*, *B. stearothermophilus*, *B. clausii*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus* and *B. thuringiensis*. The present invention also provides altered *Bacillus* strains produced using the methods described herein.

The present invention also provides methods for obtaining a protein of interest from a *Bacillus* strain comprising: transforming a *Bacillus* host cell with a DNA construct comprising a selective marker and an inactivating chromosomal segment, wherein the DNA construct is integrated into the chromosome of the *Bacillus* strain resulting in deletion of an indigenous chromosomal region or fragment thereof, to produce an altered *Bacillus* strain, culturing the altered *Bacillus* strain under suitable growth conditions to allow the expression of a protein of interest, and recovering the protein of interest. In some preferred embodiments, the protein of interest is an enzyme. In some particularly

- 12 -

preferred embodiments, the *Bacillus* host comprises a heterologous gene encoding a protein of interest. In additional embodiments, the *Bacillus* host cell is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. brevis*, *B. stearothermophilus*, *B. clausii*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus* and *B. thuringiensis*. In some preferred embodiments, the indigenous chromosomal region is selected from the group of regions consisting of PBSX, skin, prophage 7, SP β , prophage 1, prophage 2, prophage 3, prophage 4, prophage 5, prophage 6, PPS, PKS, YVFF-YVEK, DHB and fragments thereof. In some particularly preferred embodiments the altered *Bacillus* strains further comprise at least one mutation in a gene selected from the group consisting of *degU*, *degQ*, *degS*, *sco4*, *spolIE* and *oppA*. In some embodiments, the protein of interest is an enzyme selected from the group consisting of proteases, cellulases, amylases, carbohydrases, lipases, isomerases, transferases, kinases, and phosphatases. In some particularly preferred embodiments, the enzyme is a protease. In some preferred embodiments, the protein of interest is an enzyme. In other embodiments, the protein of interest is selected from the group consisting of antibodies, hormones and growth factors.

The present invention further provides methods for enhancing the expression of a protein of interest in *Bacillus* comprising: obtaining nucleic acid from at least one *Bacillus* cell; performing transcriptome DNA array analysis on the nucleic acid from said *Bacillus* cell to identify at least one gene of interest; modifying at least one gene of interest to produce a DNA construct; introducing the DNA construct into a *Bacillus* host cell to produce an altered *Bacillus* strain, wherein the altered *Bacillus* strain is capable of producing a protein of interest, under conditions such that expression of the protein of interest is enhanced as compared to the expression of the protein of interest in a *Bacillus* that has not been altered. In some embodiments, the protein of interest is associated with at least one biochemical pathway selected from the group consisting of amino acid biosynthetic pathways and biodegradative pathways. In some embodiments, the methods involve disabling at least one biodegradative pathway. In some embodiments, the biodegradative pathway is disabled due to the transcription of the gene of interest. However, it is not intended that the present invention be limited to these pathways, as it is contemplated that the methods will find use in the modification of other biochemical pathways within cells such that enhanced expression of a protein of interest results. In some particularly preferred embodiments, the *Bacillus* host comprises a heterologous gene encoding a protein of interest. In additional embodiments, the *Bacillus* host cell is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. brevis*, *B. stearothermophilus*, *B. clausii*, *B. alkalophilus*, *B.*

- 13 -

coagulans, *B. circulans*, *B. pumilus* and *B. thuringiensis*. In some embodiments, the protein of interest is an enzyme. In some preferred embodiments, the protein of interest is selected from proteases, cellulases, amylases, carbohydrases, lipases, isomerases, transferases, kinases and phosphatases, while in other embodiments, the protein of interest is selected from the group consisting of antibodies, hormones and growth factors.

The present invention further provides methods for enhancing the expression of a protein of interest in *Bacillus*, comprising: obtaining nucleic acid containing at least one gene of interest from at least one *Bacillus* cell; fragmenting said nucleic acid; amplifying said fragments to produce a pool of amplified fragments comprising said at least one gene of interest; ligating said amplified fragments to produce a DNA construct; directly transforming said DNA construct into a *Bacillus* host cell to produce an altered *Bacillus* strain; culturing said altered *Bacillus* strain under conditions such that expression of said protein of interest is enhanced as compared to the expression of said protein of interest in a *Bacillus* that has not been altered. In some preferred embodiments, said amplifying comprises using the polymerase chain reaction. In some embodiments, the altered *Bacillus* strain comprises modified gene selected from the group consisting of *prpC*, *sigD* and *tdh/kbl*. In some particularly preferred embodiments, the *Bacillus* host comprises a heterologous gene encoding a protein of interest. In additional embodiments, the *Bacillus* host cell is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. brevis*, *B. stearothermophilus*, *B. clausii*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus* and *B. thuringiensis*. In some embodiments, the protein of interest is an enzyme. In some preferred embodiments, the protein of interest is selected from proteases, cellulases, amylases, carbohydrases, lipases, isomerases, transferases, kinases and phosphatases, while in other embodiments, the protein of interest is selected from the group consisting of antibodies, hormones and growth factors.

The present invention further provides isolated nucleic acids comprising the sequences set forth in nucleic acid sequences selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 37, SEQ ID NO: 25, SEQ ID NO: 21, SEQ ID NO: 50, SEQ ID NO: 23, SEQ ID NO: 27, SEQ ID NO: 19, SEQ ID NO: 31, SEQ ID NO: 48, SEQ ID NO: 46, SEQ ID NO: 35, and SEQ ID NO: 33.

The present invention also provides isolated nucleic acid sequences encoding amino acids, wherein the amino acids are selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14,

- 14 -

SEQ ID NO: 16, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:38, SEQ ID NO:26, SEQ ID NO:22, SEQ ID NO:57, SEQ ID NO:24, SEQ ID NO:28, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:55, SEQ ID NO:53, SEQ ID NO:36, and SEQ ID NO:34.

5 The present invention further provides isolated amino acid sequences, wherein the amino acid sequences are selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:38, SEQ ID NO:26, SEQ ID NO:22, SEQ ID NO:57, 10 SEQ ID NO:24, SEQ ID NO:28, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:55, SEQ ID NO:53, SEQ ID NO:36, and SEQ ID NO:34.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1, Panels A and B illustrate a general schematic diagram of one method ("Method 1" See, Example 1) provided by the present invention. In this method, flanking regions of a gene and/or an indigenous chromosomal region are amplified out of a wild-type *Bacillus* chromosome, cut with restriction enzymes (including at least *Bam*HI) and ligated into pJM102. The construct is cloned through *E. coli* and the plasmid is isolated, 20 linearized with *Bam*HI and ligated to an antimicrobial marker with complementary ends. After cloning again in *E. coli*, a liquid culture is grown and used to isolate plasmid DNA for use in transforming a *Bacillus* host strain (preferably, a competent *Bacillus* host strain).

Figure 2 illustrates the location of primers used in the construction of a DNA cassette according to some embodiments of the present invention. The diagram provides 25 an explanation of the primer naming system used herein. Primers 1 and 4 are used for checking the presence of the deletion. These primers are referred to as "DeletionX-UF-chk" and "DeletionX-UR-chk-del." DeletionX-UF-chk is also used in a PCR reaction with a reverse primer inside the antimicrobial marker (Primer 11: called for example PBSX-UR-chk-Del) for a positive check of the cassette's presence in the chromosome. Primers 2 30 and 6 are used to amplify the upstream flanking region. These primers are referred to as "DeletionX-UF" and "DeletionX-UR," and contain engineered restriction sites at the black vertical bars. Primers 5 and 8 are used to amplify the downstream flanking region. These primers are referred to as "DeletionX-DF" and "DeletionX-DR." These primers may either contain engineered *Bam*HI sites for ligation and cloning, or 25 base pair tails homologous 35 to an appropriate part of the *Bacillus subtilis* chromosome for use in PCR fusion. In some

- 15 -

embodiments, primers 3 and 7 are used to fuse the cassette together in the case of those cassettes created by PCR fusion, while in other embodiments, they are used to check for the presence of the insert. These primers are referred to as "DeletionX-UF-nested" and "DeletionX-DR-nested." In some embodiments, the sequence corresponding to an 5 "antibiotic marker" is a Spc resistance marker and the region to be deleted is the CssS gene.

Figure 3 is a general schematic diagram of one method ("Method 2"; See Example 2) of the present invention. Flanking regions are engineered to include 25 bp of sequence complementary to a selective marker sequence. The selective marker sequence also 10 includes 25 bp tails that complement DNA of one flanking region. Primers near the ends of the flanking regions are used to amplify all three templates in a single reaction tube, thereby creating a fusion fragment. This fusion fragment or DNA construct is directly transformed into a competent *Bacillus* host strain.

Figure 4 provides an electrophoresis gel of *Bacillus* DHB deletion clones. Lanes 1 15 and 2 depict two strains carrying the DHB deletion amplified with primers 1 and 11, and illustrate a 1.2 kb band amplified from upstream of the inactivating chromosomal segments into the phleomycin marker. Lane 3 depicts the wild-type control for this reaction. Only non-specific amplification is observed. Lanes 4 and 5 depict the DHB deleted strains 20 amplified with primers 9 and 12. This 2 kb band amplifies through the antibiotic region to below the downstream section of the inactivated chromosomal segment. Lane 6 is the negative control for this reaction and a band is not illustrated. Lanes 7 and 8 depict the deletion strains amplified with primers 1 and 4 and the illustration confirms that the DHB region is missing. Lane 9 is the wild-type control.

Figure 5 illustrates gel electrophoresis of two clones of a production strain of 25 *Bacillus subtilis* (wild-type) wherein *s/r* is replaced with a phleomycin (phleo) marker which results in a deletion of the *s/r* gene. Lanes 1 and 2 represent the clones amplified with primers at locations 1 and 11. Lane 3 is the wild-type chromosomal DNA amplified with the same primers. A 1.2 kb band is observed for the insert. Lanes 4 and 5 represent the clones amplified with primers at locations 9 and 12. Lane 6 is the wild-type chromosomal 30 DNA amplified with the same primers. Correct transformants include a 2 kb band. Lanes 7 and 8 represent the clones amplified with primers at locations 2 and 4. Lane 9 is the wild-type chromosomal DNA amplified with the same primers. No band is observed for the deletion strains, but a band around 1 kb is observed in the wild-type. Reference is made to Figure 2 for an explanation of primer locations.

- 16 -

Figure 6 provides an electrophoresis gel of a clone of a production strain of *Bacillus subtilis* (wild-type) wherein *cssS* is inactivated by the integration of a *spc* marker into the chromosome. Lane 1 is a control without the integration and is approximately 1.5kb smaller.

5 Figure 7 provides a bar graph showing improved subtilisin secretion measured from shake flask cultures with *Bacillus subtilis* wild-type strain (unaltered) and corresponding altered *Bacillus subtilis* strains having various deletions. Protease activity (g/L) was measured after 17, 24 and 40 hours or was measured at 24 and 40 hours.

10 Figure 8 provides a bar graph showing improved protease secretion as measured from shake flask cultures in *Bacillus subtilis* wild-type strain (unaltered) and corresponding altered deletion strains (-*sbo*) and (-*slr*). Protease activity (g/L) was measured after 17, 24 and 40 hours.

15 **DESCRIPTION OF THE INVENTION**

The present invention provides cells that have been genetically manipulated to have an altered capacity to produce expressed proteins. In particular, the present invention relates to Gram-positive microorganisms, such as *Bacillus* species having enhanced expression of a protein of interest, wherein one or more chromosomal genes 20 have been inactivated or otherwise modified. In some preferred embodiments, one or more chromosomal genes have been deleted from the *Bacillus* chromosome. In some further embodiments, one or more indigenous chromosomal regions have been deleted from a corresponding wild-type *Bacillus* host chromosome.

25 **Definitions**

All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference. Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention 30 belongs (See e.g., Singleton *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York [1994]; and Hale and Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY [1991], both of which provide one of skill with a general dictionary of many of the terms used herein). Although any methods and materials similar or equivalent to those described herein can be used in 35 the practice or testing of the present invention, the preferred methods and materials are

- 17 -

described. Numeric ranges are inclusive of the numbers defining the range. As used herein and in the appended claims, the singular "a", "an" and "the" includes the plural reference unless the context clearly dictates otherwise. Thus, for example, reference to a "host cell" includes a plurality of such host cells.

5 Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. The headings provided herein are not limitations of the various aspects or embodiments of the invention that can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to
10 the Specification as a whole.

As used herein, "host cell" refers to a cell that has the capacity to act as a host or expression vehicle for a newly introduced DNA sequence. In preferred embodiments of the present invention, the host cells are *Bacillus* sp. or *E. coli* cells.

As used herein, "the genus *Bacillus*" includes all species within the genus
15 "*Bacillus*," as known to those of skill in the art, including but not limited to *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. clausii*, *B. halodurans*, *B. megaterium*, *B. coagulans*, *B. circulans*, *B. lautus*, and *B. thuringiensis*. It is recognized that the genus *Bacillus* continues to undergo
20 taxonomical reorganization. Thus, it is intended that the genus include species that have been reclassified, including but not limited to such organisms as *B. stearothermophilus*, which is now named "*Geobacillus stearothermophilus*." The production of resistant endospores in the presence of oxygen is considered the defining feature of the genus
25 *Bacillus*, although this characteristic also applies to the recently named *Alicyclobacillus*, *Amphibacillus*, *Aneurinibacillus*, *Anoxybacillus*, *Brevibacillus*, *Filobacillus*, *Gracilibacillus*, *Halobacillus*, *Paenibacillus*, *Salibacillus*, *Thermobacillus*, *Ureibacillus*, and *Virgibacillus*.

As used herein, "nucleic acid" refers to a nucleotide or polynucleotide sequence, and fragments or portions thereof, as well as to DNA, cDNA, and RNA of genomic or synthetic origin which may be double-stranded or single-stranded, whether representing the sense or antisense strand. It will be understood that as a result of the degeneracy of
30 the genetic code, a multitude of nucleotide sequences may encode a given protein.

As used herein the term "gene" means a chromosomal segment of DNA involved in producing a polypeptide chain that may or may not include regions preceding and following the coding regions (e.g. 5' untranslated (5' UTR) or leader sequences and 3'
35 untranslated (3' UTR) or trailer sequences, as well as intervening sequence (introns) between individual coding segments (exons)).

- 18 -

In some embodiments, the gene encodes therapeutically significant proteins or peptides, such as growth factors, cytokines, ligands, receptors and inhibitors, as well as vaccines and antibodies. The gene may encode commercially important industrial proteins or peptides, such as enzymes (e.g., proteases, carbohydrases such as amylases and glucoamylases, cellulases, oxidases and lipases). However, it is not intended that the present invention be limited to any particular enzyme or protein. In some embodiments, the gene of interest is a naturally-occurring gene, while in other embodiments, it is a mutated gene or a synthetic gene.

As used herein, the term "vector" refers to any nucleic acid that can be replicated in cells and can carry new genes or DNA segments into cells. Thus, the term refers to a nucleic acid construct designed for transfer between different host cells. An "expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art.

As used herein, the terms "DNA construct," "expression cassette," and "expression vector," refer to a nucleic acid construct generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell (i.e., these are vectors or vector elements, as described above). The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter. In some embodiments, DNA constructs also include a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. In one embodiment, a DNA construct of the invention comprises a selective marker and an inactivating chromosomal segment as defined herein.

As used herein, "transforming DNA," "transforming sequence," and "DNA construct" refer to DNA that is used to introduce sequences into a host cell or organism. Transforming DNA is DNA used to introduce sequences into a host cell or organism. The DNA may be generated *in vitro* by PCR or any other suitable techniques. In some preferred embodiments, the transforming DNA comprises an incoming sequence, while in other preferred embodiments it further comprise an incoming sequence flanked by homology boxes. In yet a further embodiment, the transforming DNA comprises other non-homologous sequences, added to the ends (i.e., stuffer sequences or flanks). The

- 19 -

ends can be closed such that the transforming DNA forms a closed circle, such as, for example, insertion into a vector.

As used herein, the term "plasmid" refers to a circular double-stranded (ds) DNA construct used as a cloning vector, and which forms an extrachromosomal self-replicating 5 genetic element in many bacteria and some eukaryotes. In some embodiments, plasmids become incorporated into the genome of the host cell.

As used herein, the terms "isolated" and "purified" refer to a nucleic acid or amino acid (or other component) that is removed from at least one component with which it is naturally associated.

10 As used herein, the term "enhanced expression" is broadly construed to include enhanced production of a protein of interest. Enhanced expression is that expression above the normal level of expression in the corresponding host strain that has not been altered according to the teachings herein but has been grown under essentially the same growth conditions.

15 In some preferred embodiments, "enhancement" is achieved by any modification that results in an increase in a desired property. For example, in some particularly preferred embodiments, the present invention provides means for enhancing protein production, such that the enhanced strains produced a greater quantity and/or quality of a protein of interest than the parental strain (e.g., the wild-type and/or originating strain).

20 As used herein the term "expression" refers to a process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

25 As used herein in the context of introducing a nucleic acid sequence into a cell, the term "introduced" refers to any method suitable for transferring the nucleic acid sequence into the cell. Such methods for introduction include but are not limited to protoplast fusion, transfection, transformation, conjugation, and transduction (See e.g., Ferrari et al., "Genetics," in Hardwood et al, (eds.), *Bacillus*, Plenum Publishing Corp., pages 57-72, [1989]).

30 As used herein, the terms "transformed" and "stably transformed" refers to a cell that has a non-native (heterologous) polynucleotide sequence integrated into its genome or as an episomal plasmid that is maintained for at least two generations.

35 As used herein "an incoming sequence" refers to a DNA sequence that is introduced into the *Bacillus* chromosome. In some preferred embodiments, the incoming sequence is part of a DNA construct. In preferred embodiments, the incoming sequence encodes one or more proteins of interest. In some embodiments, the incoming sequence comprises a

- 20 -

sequence that may or may not already be present in the genome of the cell to be transformed (i.e., it may be either a homologous or heterologous sequence). In some embodiments, the incoming sequence encodes one or more proteins of interest, a gene, and/or a mutated or modified gene. In alternative embodiments, the incoming sequence 5 encodes a functional wild-type gene or operon, a functional mutant gene or operon, or a non-functional gene or operon. In some embodiments, the non-functional sequence may be inserted into a gene to disrupt function of the gene. In some embodiments, the incoming sequence encodes one or more functional wild-type genes, while in other embodiments, the incoming sequence encodes one or more functional mutant genes, and in yet additional 10 embodiments, the incoming sequence encodes one or more non-functional genes. In another embodiment, the incoming sequence encodes a sequence that is already present in the chromosome of the host cell to be transformed. In a preferred embodiment, the incoming sequence comprises a gene selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spollSA*, *phrC*, *sigB*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *pmpC*, 15 *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*, and fragments thereof. In yet another embodiment, the incoming sequence includes a selective marker. In a further embodiment the incoming sequence includes two homology boxes.

In some embodiments, the incoming sequence encodes at least one heterologous protein including, but not limited to hormones, enzymes, and growth factors. In another 20 embodiment, the enzyme includes, but is not limited to hydrolases, such as protease, esterase, lipase, phenol oxidase, permease, amylase, pullulanase, cellulase, glucose isomerase, laccase and protein disulfide isomerase.

As used herein, "homology box" refers to a nucleic acid sequence, which is homologous to a sequence in the *Bacillus* chromosome. More specifically, a homology 25 box is an upstream or downstream region having between about 80 and 100% sequence identity, between about 90 and 100% sequence identity, or between about 95 and 100% sequence identity with the immediate flanking coding region of a gene or part of a gene to be inactivated according to the invention. These sequences direct where in the *Bacillus* chromosome a DNA construct is integrated and directs what part of the *Bacillus* 30 chromosome is replaced by the incoming sequence. While not meant to limit the invention, a homology box may include about between 1 base pair (bp) to 200 kilobases (kb). Preferably, a homology box includes about between 1 bp and 10.0 kb; between 1 bp and 5.0 kb; between 1 bp and 2.5 kb; between 1 bp and 1.0 kb, and between 0.25 kb and 2.5 kb. A homology box may also include about 10.0 kb, 5.0 kb, 2.5 kb, 2.0 kb, 1.5 kb, 1.0 35 kb, 0.5 kb, 0.25 kb and 0.1 kb. In some embodiments, the 5' and 3' ends of a selective

- 21 -

marker are flanked by a homology box wherein the homology box comprises nucleic acid sequences immediately flanking the coding region of the gene.

As used herein, the term "selectable marker-encoding nucleotide sequence" refers to a nucleotide sequence which is capable of expression in the host cells and where 5 expression of the selectable marker confers to cells containing the expressed gene the ability to grow in the presence of a corresponding selective agent or lack of an essential nutrient.

As used herein, the terms "selectable marker" and "selective marker" refer to a nucleic acid (e.g., a gene) capable of expression in host cell which allows for ease of 10 selection of those hosts containing the vector. Examples of such selectable markers include but are not limited to antimicrobials. Thus, the term "selectable marker" refers to genes that provide an indication that a host cell has taken up an incoming DNA of interest or some other reaction has occurred. Typically, selectable markers are genes that confer antimicrobial resistance or a metabolic advantage on the host cell to allow cells containing 15 the exogenous DNA to be distinguished from cells that have not received any exogenous sequence during the transformation. A "residing selectable marker" is one that is located on the chromosome of the microorganism to be transformed. A residing selectable marker encodes a gene that is different from the selectable marker on the transforming DNA construct. Selective markers are well known to those of skill in the art. As indicated 20 above, preferably the marker is an antimicrobial resistant marker (e.g., amp^R; phleo^R; spec^R; kan^R; ery^R; tet^R; cmp^R; and neo^R; See e.g., Guerot-Fleury, Gene, 167:335-337 [1995]; Palmeros et al., Gene 247:255-264 [2000]; and Trieu-Cuot et al., Gene, 23:331-341 [1983]). In some particularly preferred embodiments, the present invention provides a 25 chloramphenicol resistance gene (e.g., the gene present on pC194, as well as the resistance gene present in the *Bacillus licheniformis* genome). This resistance gene is particularly useful in the present invention, as well as in embodiments involving chromosomal amplification of chromosomally integrated cassettes and integrative plasmids (See e.g., Albertini and Galizzi, Bacteriol., 162:1203-1211 [1985]; and Stahl and Ferrari, J. Bacteriol., 158:411-418 [1984]). The DNA sequence of this naturally-occurring 30 chloramphenicol resistance gene is shown below:

ATGAATTTCAAACAATCGAGCTTGACACATGGTATAGAAAATCTTATTTGACCATT
CATGAAGGAAGCGAAATGTTCTTCAGCATCACGGCAAACGTCATGTGACAAATTG
CTCGCCGTGCTCAAGAAAAAGAAGCTCAAGCTGTATCCGGCTTTATTATATCGTAT
35 CAAGGGTCATTCAATTGCCCTGAGTTAGAACACGTTGATGACAAAGGAAGCT
GGGTTATTGGGAACAAATGCATCCGTGCTATGCGATTTTATCAGGACGACCAAAAC
GTTTCCGCCCTCTGGACGGAATACTCAGACGATTTTCGCAGTTTATCATCAATAT

- 22 -

5 CTTCTGGACGCCGAGCGCTTGGAGACAAAAGGGGCCTTGGGCTAACGCCGGACAT
CCCGCCAATACGTTTCAGTTCTATTCCATGGGTGCGCTTTCAACATTCAATT
TAAACCTTGATAACAGCGAACACTGCTGCCGATTATTACAAACGGGAAACTTTC
AGAAGGCAGGGAAACATTGGCCGTTCTGCAAGTTCACCATGCAGTGTGTGAC
GGCTATCATGCCGGCGCTTTATAA (SEQ ID NO:58).

The deduced amino acid sequence of this chloramphenicol resistance protein is:
10 MNFQTIELDTWYRKSYFDHYMKEAKCSFSITANVNTNLLAVLKKKKLKLYPAFIYIVSRVI
HSRPEFRRTFDDKGQLGYWEQMHPCYAIFHQDDQTFSALWTEYSDDFSQFYHQYLLDA
ERFGDKRGLWAKPDIPPNTFSVSSIPWVRFSTFNLDNSEHLLPIITNGKYFSEGRETFL
PVSKFTMQCVTAIMPALL (SEQ ID NO:59).

Other markers useful in accordance with the invention include, but are not limited
to auxotrophic markers, such as tryptophan; and detection markers, such as β -
15 galactosidase.

As used herein, the term "promoter" refers to a nucleic acid sequence that
functions to direct transcription of a downstream gene. In preferred embodiments, the
promoter is appropriate to the host cell in which the target gene is being expressed. The
promoter, together with other transcriptional and translational regulatory nucleic acid
20 sequences (also termed "control sequences") is necessary to express a given gene. In
general, the transcriptional and translational regulatory sequences include, but are not
limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop
sequences, translational start and stop sequences, and enhancer or activator sequences.

A nucleic acid is "operably linked" when it is placed into a functional relationship
25 with another nucleic acid sequence. For example, DNA encoding a secretory leader (*i.e.*,
a signal peptide), is operably linked to DNA for a polypeptide if it is expressed as a
preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is
operably linked to a coding sequence if it affects the transcription of the sequence; or a
30 ribosome binding site is operably linked to a coding sequence if it is positioned so as to
facilitate translation. Generally, "operably linked" means that the DNA sequences being
linked are contiguous, and, in the case of a secretory leader, contiguous and in reading
phase. However, enhancers do not have to be contiguous. Linking is accomplished by
ligation at convenient restriction sites. If such sites do not exist, the synthetic
oligonucleotide adaptors or linkers are used in accordance with conventional practice.

35 The term "inactivation" includes any method that prevents the functional
expression of one or more of the *sbo*, *slr*, *ybcO*, *csn*, *spoIIIA*, *sigB*, *phrC*, *rapA*, *CssS*,
trpA, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*,
ycgM, *rocF*, and *rocD* chromosomal genes, wherein the gene or gene product is unable to
exert its known function. Inactivation or enhancement occurs via any suitable means,

- 23 -

including deletions, substitutions (e.g., mutations), interruptions, and/or insertions in the nucleic acid gene sequence. In one embodiment, the expression product of an inactivated gene is a truncated protein with a corresponding change in the biological activity of the protein. In some embodiments, the change in biological activity is an increase in activity, 5 while in preferred embodiments, the change results in the loss of biological activity. In some embodiments, an altered *Bacillus* strain comprises inactivation of one or more genes that results preferably in stable and non-reverting inactivation.

In some preferred embodiments, inactivation is achieved by deletion. In some preferred embodiments, the gene is deleted by homologous recombination. For example, 10 in some embodiments when *sbo* is the gene to be deleted, a DNA construct comprising an incoming sequence having a selective marker flanked on each side by a homology box is used. The homology box comprises nucleotide sequences homologous to nucleic acids flanking regions of the chromosomal *sbo* gene. The DNA construct aligns with the homologous sequences of the *Bacillus* host chromosome and in a double crossover event 15 the *sbo* gene is excised out of the host chromosome.

As used herein, "deletion" of a gene refers to deletion of the entire coding sequence, deletion of part of the coding sequence, or deletion of the coding sequence including flanking regions. The deletion may be partial as long as the sequences left in the chromosome provides the desired biological activity of the gene. The flanking regions 20 of the coding sequence may include from about 1bp to about 500 bp at the 5' and 3' ends. The flanking region may be larger than 500 bp but will preferably not include other genes in the region which may be inactivated or deleted according to the invention. The end result is that the deleted gene is effectively non-functional. In simple terms, a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more 25 nucleotides or amino acid residues, respectively, have been removed (i.e., are absent). Thus, a "deletion mutant" has fewer nucleotides or amino acids than the respective wild-type organism.

In still another embodiment of the present invention, deletion of a gene active at an inappropriate time as determined by DNA array analysis (e.g., transcriptome analysis, as 30 described herein) provides enhanced expression of a product protein. In some preferred embodiments, deletion of one or more of genes selected from the group consisting of *pckA*, *gapB*, *fbp*, and/or *alsD*, provides an improved strain for the improved efficiency of feed utilization. As used herein, "transcriptome analysis" refers to the analysis of gene transcription.

- 24 -

In another embodiment of the present invention, a gene is considered to be "optimized" by the deletion of a regulatory sequence in which this deletion results in increased expression of a desired product. In some preferred embodiments of the present invention, the tryptophan operon (*i.e.*, comprising genes *trpA* *trpB*, *trpC*, *trpD*, *trpE*, *trpF*) 5 is optimized by the deletion of the DNA sequence coding for the TRAP binding RNA sequence (See, Yang, *et. al.*, *J Mol. Biol.*, 270:696-710 [1997]). This deletion is contemplated to increase expression of the desired product from the host strain.

In another preferred embodiment, inactivation is by insertion. For example, in some embodiments, when *sbo* is the gene to be inactivated, a DNA construct comprises 10 an incoming sequence having the *sbo* gene interrupted by a selective marker. The selective marker will be flanked on each side by sections of the *sbo* coding sequence. The DNA construct aligns with essentially identical sequences of the *sbo* gene in the host chromosome and in a double crossover event the *sbo* gene is inactivated by the insertion of the selective marker. In simple terms, an "insertion" or "addition" is a change in a 15 nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring sequence.

In another embodiment, activation is by insertion in a single crossover event with a plasmid as the vector. For example, a *sbo* chromosomal gene is aligned with a plasmid 20 comprising the gene or part of the gene coding sequence and a selective marker. In some embodiments, the selective marker is located within the gene coding sequence or on a part of the plasmid separate from the gene. The vector is integrated into the *Bacillus* chromosome, and the gene is inactivated by the insertion of the vector in the coding sequence.

25 In alternative embodiments, inactivation results due to mutation of the gene. Methods of mutating genes are well known in the art and include but are not limited to site-directed mutation, generation of random mutations, and gapped-duplex approaches (See e.g., U.S. Pat. 4,760,025; Moring *et al.*, *Biotech.* 2:646 [1984]; and Kramer *et al.*, *Nucleic Acids Res.*, 12:9441 [1984]).

30 As used herein, a "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

As used herein, "homologous genes" refers to a pair of genes from different, but usually related species, which correspond to each other and which are identical or very similar to each other. The term encompasses genes that are separated by speciation (*i.e.*,

- 25 -

the development of new species) (e.g., orthologous genes), as well as genes that have been separated by genetic duplication (e.g., paralogous genes).

As used herein, "ortholog" and "orthologous genes" refer to genes in different species that have evolved from a common ancestral gene (i.e., a homologous gene) by speciation. Typically, orthologs retain the same function in during the course of evolution. Identification of orthologs finds use in the reliable prediction of gene function in newly sequenced genomes.

As used herein, "paralog" and "paralogous genes" refer to genes that are related by duplication within a genome. While orthologs retain the same function through the course of evolution, paralogs evolve new functions, even though some functions are often related to the original one. Examples of paralogous genes include, but are not limited to genes encoding trypsin, chymotrypsin, elastase, and thrombin, which are all serine proteinases and occur together within the same species.

As used herein, "homology" refers to sequence similarity or identity, with identity being preferred. This homology is determined using standard techniques known in the art (See e.g., Smith and Waterman, *Adv. Appl. Math.*, 2:482 [1981]; Needleman and Wunsch, *J. Mol. Biol.*, 48:443 [1970]; Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444 [1988]; programs such as GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Genetics Computer Group, Madison, WI); and Devereux *et al.*, *Nucl. Acid Res.*, 12:387-395 [1984]).

As used herein, an "analogous sequence" is one wherein the function of the gene is essentially the same as the gene designated from *Bacillus subtilis* strain 168. Additionally, analogous genes include at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% sequence identity with the sequence of the *Bacillus subtilis* strain 168 gene. Alternately, analogous sequences have an alignment of between 70 to 100% of the genes found in the *B. subtilis* 168 region and/or have at least between 5 - 10 genes found in the region aligned with the genes in the *B. subtilis* 168 chromosome. In additional embodiments more than one of the above properties applies to the sequence. Analogous sequences are determined by known methods of sequence alignment. A commonly used alignment method is BLAST, although as indicated above and below, there are other methods that also find use in aligning sequences.

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and

- 26 -

Doolittle (Feng and Doolittle, J. Mol. Evol., 35:351-360 [1987]). The method is similar to that described by Higgins and Sharp (Higgins and Sharp, CABIOS 5:151-153 [1989]). Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

5 Another example of a useful algorithm is the BLAST algorithm, described by Altschul *et al.*, (Altschul *et al.*, J. Mol. Biol., 215:403-410, [1990]; and Karlin *et al.*, Proc. Natl. Acad. Sci. USA 90:5873-5787 [1993]). A particularly useful BLAST program is the WU-BLAST-2 program (See, Altschul *et al.*, Meth. Enzymol., 266:460-480 [1996]). WU-BLAST-2 uses several search parameters, most of which are set to the default values.

10 The adjustable parameters are set with the following values: overlap span =1, overlap fraction = 0.125, word threshold (T) = 11. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. However, the values may be adjusted to increase

15 sensitivity. A % amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

20 Thus, "percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues of the sequence shown in the nucleic acid figures. A preferred method utilizes the BLASTN module of WU-BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

25 The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer nucleosides than those of the nucleic acid figures, it is understood that the percentage of homology will be determined based on the number of homologous nucleosides in relation to the total number of nucleosides. Thus, for example, homology of sequences shorter than those of

30 the sequences identified herein and as discussed below, will be determined using the number of nucleosides in the shorter sequence.

As used herein, the term "hybridization" refers to the process by which a strand of nucleic acid joins with a complementary strand through base pairing, as known in the art.

35 A nucleic acid sequence is considered to be "selectively hybridizable" to a reference nucleic acid sequence if the two sequences specifically hybridize to one another

- 27 -

- under moderate to high stringency hybridization and wash conditions. Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex or probe. For example, "maximum stringency" typically occurs at about Tm-5°C (5° below the Tm of the probe); "high stringency" at about 5-10°C below the Tm; "intermediate stringency" at about 10-20°C below the Tm of the probe; and "low stringency" at about 20-25°C below the Tm. Functionally, maximum stringency conditions may be used to identify sequences having strict identity or near-strict identity with the hybridization probe; while an intermediate or low stringency hybridization can be used to identify or detect polynucleotide sequence homologs.
- 10 Moderate and high stringency hybridization conditions are well known in the art. An example of high stringency conditions includes hybridization at about 42°C in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured carrier DNA followed by washing two times in 2X SSC and 0.5% SDS at room temperature and two additional times in 0.1X SSC and 0.5% SDS at 42°C. An example of moderate
- 15 stringent conditions include an overnight incubation at 37°C in a solution comprising 20% formamide, 5 x SSC (150mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1x SSC at about 37 - 50°C. Those of skill in the art know how to adjust the temperature, ionic strength, etc. as necessary to
- 20 accommodate factors such as probe length and the like.

As used herein, "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid sequence or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention. "Recombination, "recombining," or generating a "recombined" nucleic acid is generally the assembly of two or more nucleic acid fragments wherein the assembly gives rise to a chimeric gene.

In a preferred embodiment, mutant DNA sequences are generated with site saturation mutagenesis in at least one codon. In another preferred embodiment, site saturation mutagenesis is performed for two or more codons. In a further embodiment, mutant DNA sequences have more than 40%, more than 45%, more than 50%, more than 55%, more than 60%, more than 65%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 95%, or more than 98% homology with the wild-type sequence. In alternative embodiments, mutant DNA is generated *in vivo* using

- 28 -

any known mutagenic procedure such as, for example, radiation, nitrosoguanidine and the like. The desired DNA sequence is then isolated and used in the methods provided herein.

In an alternative embodiment, the transforming DNA sequence comprises
5 homology boxes without the presence of an incoming sequence. In this embodiment, it is desired to delete the endogenous DNA sequence between the two homology boxes. Furthermore, in some embodiments, the transforming sequences are wild-type, while in other embodiments, they are mutant or modified sequences. In addition, in some embodiments, the transforming sequences are homologous, while in other embodiments,
10 they are heterologous.

As used herein, the term "target sequence" refers to a DNA sequence in the host cell that encodes the sequence where it is desired for the incoming sequence to be inserted into the host cell genome. In some embodiments, the target sequence encodes a functional wild-type gene or operon, while in other embodiments the target sequence encodes a functional
15 mutant gene or operon, or a non-functional gene or operon.

As used herein, a "flanking sequence" refers to any sequence that is either upstream or downstream of the sequence being discussed (e.g., for genes A-B-C, gene B is flanked by the A and C gene sequences). In a preferred embodiment, the incoming sequence is flanked by a homology box on each side. In another embodiment, the
20 incoming sequence and the homology boxes comprise a unit that is flanked by stuffer sequence on each side. In some embodiments, a flanking sequence is present on only a single side (either 3' or 5'), but in preferred embodiments, it is on each side of the sequence being flanked. The sequence of each homology box is homologous to a sequence in the *Bacillus* chromosome. These sequences direct where in the *Bacillus*
25 chromosome the new construct gets integrated and what part of the *Bacillus* chromosome will be replaced by the incoming sequence. In a preferred embodiment, the 5' and 3' ends of a selective marker are flanked by a polynucleotide sequence comprising a section of the inactivating chromosomal segment. In some embodiments, a flanking sequence is present on only a single side (either 3' or 5'), while in preferred embodiments, it is present
30 on each side of the sequence being flanked.

As used herein, the term "stuffer sequence" refers to any extra DNA that flanks homology boxes (typically vector sequences). However, the term encompasses any non-homologous DNA sequence. Not to be limited by any theory, a stuffer sequence provides a noncritical target for a cell to initiate DNA uptake.

- 29 -

As used herein, the term "library of mutants" refers to a population of cells which are identical in most of their genome but include different homologues of one or more genes. Such libraries find use for example, in methods to identify genes or operons with improved traits.

5 As used herein, the terms "hypercompetent" and "super competent" mean that greater than 1% of a cell population is transformable with chromosomal DNA (e.g., *Bacillus* DNA). Alternatively, the terms are used in reference to cell populations in which greater than 10% of a cell population is transformable with a self-replicating plasmid (e.g., a *Bacillus* plasmid). Preferably, the super competent cells are transformed at a rate greater than
10 observed for the wild-type or parental cell population. Super competent and hypercompetent are used interchangeably herein.

As used herein, the terms "amplification" and "gene amplification" refer to a process by which specific DNA sequences are disproportionately replicated such that the amplified gene becomes present in a higher copy number than was initially present in the genome. In some embodiments, selection of cells by growth in the presence of a drug (e.g., an inhibitor of an inhibitable enzyme) results in the amplification of either the endogenous gene encoding the gene product required for growth in the presence of the drug or by amplification of exogenous (*i.e.*, input) sequences encoding this gene product, or both.
15

20 "Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (*i.e.*, replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (*i.e.*, synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is
25 frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid. Amplification techniques have been designed primarily for this sorting out.

As used herein, the term "co-amplification" refers to the introduction into a single cell of an amplifiable marker in conjunction with other gene sequences (*i.e.*, comprising
30 one or more non-selectable genes such as those contained within an expression vector) and the application of appropriate selective pressure such that the cell amplifies both the amplifiable marker and the other, non-selectable gene sequences. The amplifiable marker may be physically linked to the other gene sequences or alternatively two separate pieces of DNA, one containing the amplifiable marker and the other containing the non-selectable
35 marker, may be introduced into the same cell.

- 30 -

As used herein, the terms "amplifiable marker," "amplifiable gene," and "amplification vector" refer to a gene or a vector encoding a gene which permits the amplification of that gene under appropriate growth conditions.

"Template specificity" is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid. For example, in the case of Q β replicase, MDV-1 RNA is the specific template for the replicase (See e.g., Kacian et al., Proc. Natl. Acad. Sci. USA 69:3038 [1972]). Other nucleic acids are not replicated by this amplification enzyme. Similarly, in the case of T7 RNA polymerase, this amplification enzyme has a stringent specificity for its own promoters (See, Chamberlin et al., Nature 228:227 [1970]). In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides or polynucleotides, where there is a mismatch between the oligonucleotide or polynucleotide substrate and the template at the ligation junction (See, Wu and Wallace, Genomics 4:560 [1989]). Finally, *Taq* and *Pfu* polymerases, by virtue of their ability to function at high temperature, are found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer hybridization with the target sequences and not hybridization with non-target sequences.

As used herein, the term "amplifiable nucleic acid" refers to nucleic acids which may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

As used herein, the term "sample template" refers to nucleic acid originating from a sample which is analyzed for the presence of "target" (defined below). In contrast, "background template" is used in reference to nucleic acid other than sample template which may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (i.e., in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum

efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labeled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

As used herein, the term "target," when used in reference to the polymerase chain reaction, refers to the region of nucleic acid bounded by the primers used for polymerase chain reaction. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

As used herein, the term "polymerase chain reaction" ("PCR") refers to the methods of U.S. Patent Nos. 4,683,195 4,683,202, and 4,965,188, hereby incorporated by reference, which include methods for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment

- 32 -

of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target 5 sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

As used herein, the term "amplification reagents" refers to those reagents (deoxyribonucleotide triphosphates, buffer, etc.), needed for amplification except for primers, nucleic acid template and the amplification enzyme. Typically, amplification 10 reagents along with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (e.g., hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme 15 conjugate detection; incorporation of ³²P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide or polynucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

20 As used herein, the terms "PCR product," "PCR fragment," and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

25 As used herein, the term "RT-PCR" refers to the replication and amplification of RNA sequences. In this method, reverse transcription is coupled to PCR, most often using a one enzyme procedure in which a thermostable polymerase is employed, as described in U.S. Patent No. 5,322,770, herein incorporated by reference. In RT-PCR, the RNA template is converted to cDNA due to the reverse transcriptase activity of the 30 polymerase, and then amplified using the polymerizing activity of the polymerase (*i.e.*, as in other PCR methods).

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

- 33 -

A "restriction site" refers to a nucleotide sequence recognized and cleaved by a given restriction endonuclease and is frequently the site for insertion of DNA fragments. In certain embodiments of the invention restriction sites are engineered into the selective marker and into 5' and 3' ends of the DNA construct.

5 As used herein "an inactivating chromosomal segment" comprises two sections. Each section comprises polynucleotides that are homologous with the upstream or downstream genomic chromosomal DNA that immediately flanks an indigenous chromosome region as defined herein. "Immediately flanks" means the nucleotides comprising the inactivating chromosomal segment do not include the nucleotides defining
10 the indigenous chromosomal region. The inactivating chromosomal segment directs where in the *Bacillus* chromosome the DNA construct gets integrated and what part of the *Bacillus* chromosome will be replaced.

As used herein, "indigenous chromosomal region" and "a fragment of an indigenous chromosomal region" refer to a segment of the *Bacillus* chromosome which is deleted from a *Bacillus* host cell in some embodiments of the present invention. In
15 general, the terms "segment," "region," "section," and "element" are used interchangeably herein. In some embodiments, deleted segments include one or more genes with known functions, while in other embodiments, deleted segments include one or more genes with unknown functions, and in other embodiments, the deleted segments include a
20 combination of genes with known and unknown functions. In some embodiments, indigenous chromosomal regions or fragments thereof include as many as 200 genes or more.

In some embodiments, an indigenous chromosomal region or fragment thereof has a necessary function under certain conditions, but the region is not necessary for *Bacillus*
25 strain viability under laboratory conditions. Preferred laboratory conditions include but are not limited to conditions such as growth in a fermenter, in a shake flask on plated media, etc., at standard temperatures and atmospheric conditions (e.g., aerobic).

An indigenous chromosomal region or fragment thereof may encompass a range of about 0.5kb to 500 kb; about 1.0 kb to 500 kb; about 5 kb to 500 kb; about 10 kb to
30 500kb; about 10 kb to 200kb; about 10kb to 100kb; about 10kb to 50kb; about 100kb to 500kb; and about 200kb to 500 kb of the *Bacillus* chromosome. In another aspect, when an indigenous chromosomal region or fragment thereof has been deleted, the chromosome of the altered *Bacillus* strain may include 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 85%, 80%, 75% or 70% of the corresponding unaltered *Bacillus*
35 host chromosome. Preferably, the chromosome of an altered *Bacillus* strain according to

- 34 -

the invention will include about 99 to 90%; 99 to 92%; and 98 to 94% of the corresponding unaltered *Bacillus* host strain chromosome genome.

As used herein, "strain viability" refers to reproductive viability. The deletion of an indigenous chromosomal region or fragment thereof, does not deleteriously affect division 5 and survival of the altered *Bacillus* strain under laboratory conditions.

As used herein, "altered *Bacillus* strain" refers to a genetically engineered *Bacillus* sp. wherein a protein of interest has an enhanced level of expression and/or production as compared to the expression and/or production of the same protein of interest in a corresponding unaltered *Bacillus* host strain grown under essentially the same growth 10 conditions. In some embodiments, the enhanced level of expression results from the inactivation of one or more chromosomal genes. In one embodiment, the enhanced level of expression results from the deletion of one or more chromosomal genes. In some embodiments, the altered *Bacillus* strains are genetically engineered *Bacillus* sp. having one or more deleted indigenous chromosomal regions or fragments thereof, wherein a 15 protein of interest has an enhanced level of expression or production, as compared to a corresponding unaltered *Bacillus* host strain grown under essentially the same growth conditions. In an alternative embodiment, the enhanced level of expression results from the insertional inactivation of one or more chromosomal genes. In some alternate embodiments, enhanced level of expression results due to increased activation or an otherwise optimized gene. In some preferred embodiments, the inactivated genes are 20 selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spollSA*, *phrC*, *sigB*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*.

In certain embodiments, the altered *Bacillus* strain comprise two inactivated 25 genes, while in other embodiments, there are three inactivated genes, four inactivated genes, five inactivated genes, six inactivated genes, or more. Thus, it is not intended that the number of inactivated genes be limited to an particular number of genes. In some embodiments, the inactivated genes are contiguous to each another, while in other 30 embodiments, they are located in separate regions of the *Bacillus* chromosome. In some embodiments, an inactivated chromosomal gene has a necessary function under certain conditions, but the gene is not necessary for *Bacillus* strain viability under laboratory conditions. Preferred laboratory conditions include but are not limited to conditions such as growth in a fermenter, in a shake flask, plated media, etc., suitable for the growth of the microorganism.

- 35 -

As used herein, a "corresponding unaltered *Bacillus* strain" is the host strain (e.g., the originating and/or wild-type strain) from which the indigenous chromosomal region or fragment thereof is deleted or modified and from which the altered strain is derived.

As used herein, the term "chromosomal integration" refers to the process whereby
5 the incoming sequence is introduced into the chromosome of a host cell (e.g., *Bacillus*). The homologous regions of the transforming DNA align with homologous regions of the chromosome. Subsequently, the sequence between the homology boxes is replaced by the incoming sequence in a double crossover (i.e., homologous recombination). In some embodiments of the present invention, homologous sections of an inactivating
10 chromosomal segment of a DNA construct align with the flanking homologous regions of the indigenous chromosomal region of the *Bacillus* chromosome. Subsequently, the indigenous chromosomal region is deleted by the DNA construct in a double crossover (i.e., homologous recombination).

"Homologous recombination" means the exchange of DNA fragments between two
15 DNA molecules or paired chromosomes at the site of identical or nearly identical nucleotide sequences. In a preferred embodiment, chromosomal integration is homologous recombination.

"Homologous sequences" as used herein means a nucleic acid or polypeptide sequence having 100%, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 88%,
20 85%, 80%, 75%, or 70% sequence identity to another nucleic acid or polypeptide sequence when optimally aligned for comparison. In some embodiments, homologous sequences have between 85% and 100% sequence identity, while in other embodiments there is between 90% and 100% sequence identity, and in more preferred embodiments, there is 95% and 100% sequence identity.

25 As used herein "amino acid" refers to peptide or protein sequences or portions thereof. The terms "protein", "peptide" and "polypeptide" are used interchangeably.

As used herein, "protein of interest" and "polypeptide of interest" refer to a protein/polypeptide that is desired and/or being assessed. In some embodiments, the protein of interest is intracellular, while in other embodiments, it is a secreted polypeptide.
30 Particularly preferred polypeptides include enzymes, including, but not limited to those selected from amylolytic enzymes, proteolytic enzymes, cellulolytic enzymes, oxidoreductase enzymes and plant cell-wall degrading enzymes. More particularly, these enzymes include, but are not limited to amylases, proteases, xylanases, lipases, laccases, phenol oxidases, oxidases, cutinases, cellulases, hemicellulases, esterases, peroxidases,
35 catalases, glucose oxidases, phytases, pectinases, glucosidases, isomerases,

- 36 -

transferases, galactosidases and chitinases. In some particularly preferred embodiments of the present invention, the polypeptide of interest is a protease. In some embodiments, the protein of interest is a secreted polypeptide which is fused to a signal peptide (*i.e.*, an amino-terminal extension on a protein to be secreted). Nearly all secreted proteins use an
5 amino-terminal protein extension which plays a crucial role in the targeting to and translocation of precursor proteins across the membrane. This extension is proteolytically removed by a signal peptidase during or immediately following membrane transfer.

In some embodiments of the present invention, the polypeptide of interest is selected from hormones, antibodies, growth factors, receptors, etc. Hormones
10 encompassed by the present invention include but are not limited to, follicle-stimulating hormone, luteinizing hormone, corticotropin-releasing factor, somatostatin, gonadotropin hormone, vasopressin, oxytocin, erythropoietin, insulin and the like. Growth factors include, but are not limited to platelet-derived growth factor, insulin-like growth factors, epidermal growth factor, nerve growth factor, fibroblast growth factor, transforming growth
15 factors, cytokines, such as interleukins (*e.g.*, IL-1 through IL-13), interferons, colony stimulating factors, and the like. Antibodies include but are not limited to immunoglobulins obtained directly from any species from which it is desirable to produce antibodies. In addition, the present invention encompasses modified antibodies. Polyclonal and
20 monoclonal antibodies are also encompassed by the present invention. In particularly preferred embodiments, the antibodies are human antibodies.

As used herein, the term "heterologous protein" refers to a protein or polypeptide that does not naturally occur in the host cell. Examples of heterologous proteins include enzymes such as hydrolases including proteases, cellulases, amylases, carbohydrases, and lipases; isomerases such as racemases, epimerases, tautomerases, or mutases; transferases,
25 kinases and phosphatases. In some embodiments, the proteins are therapeutically significant proteins or peptides, including but not limited to growth factors, cytokines, ligands, receptors and inhibitors, as well as vaccines and antibodies. In additional embodiments, the proteins are commercially important industrial proteins/peptides (*e.g.*, proteases, carbohydrates such as amylases and glucoamylases, cellulases, oxidases and lipases). In
30 some embodiments, the gene encoding the proteins are naturally occurring genes, while in other embodiments, mutated and/or synthetic genes are used.

As used herein, "homologous protein" refers to a protein or polypeptide native or naturally occurring in a cell. In preferred embodiments, the cell is a Gram-positive cell, while in particularly preferred embodiments, the cell is a *Bacillus* host cell. In alternative
35 embodiments, the homologous protein is a native protein produced by other organisms,

- 37 -

including but not limited to *E. coli*. The invention encompasses host cells producing the homologous protein via recombinant DNA technology.

As used herein, an "operon region" comprises a group of contiguous genes that are transcribed as a single transcription unit from a common promoter, and are thereby subject to co-regulation. In some embodiments, the operon includes a regulator gene. In most preferred embodiments, operons that are highly expressed as measured by RNA levels, but have an unknown or unnecessary function are used.

As used herein, a "multi-contiguous single gene region" is a region wherein at least the coding regions of two genes occur in tandem and in some embodiments, include intervening sequences preceding and following the coding regions. In some embodiments, an antimicrobial region is included.

As used herein, an "antimicrobial region" is a region containing at least one gene that encodes an antimicrobial protein.

15

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides cells that have been genetically manipulated to have an altered capacity to produce expressed proteins. In particular, the present invention relates to Gram-positive microorganisms, such as *Bacillus* species having enhanced expression of a protein of interest, wherein one or more chromosomal genes have been inactivated, and preferably wherein one or more chromosomal genes have been deleted from the *Bacillus* chromosome. In some further embodiments, one or more indigenous chromosomal regions have been deleted from a corresponding wild-type *Bacillus* host chromosome. Indeed, the present invention provides means for deletion of single or multiple genes, as well as large chromosomal deletions. In preferred embodiments, such deletions provide advantages such as improved production of a protein of interest.

A. Gene Deletions

30 As indicated above, the present invention includes embodiments that involve single or multiple gene deletions and/or mutations, as well as large chromosomal deletions.

In some preferred embodiments, the present invention includes a DNA construct comprising an incoming sequence. The DNA construct is assembled *in vitro*, followed by direct cloning of the construct into a competent *Bacillus* host, such that the DNA construct becomes integrated into the *Bacillus* chromosome. For example, PCR fusion and/or ligation can be employed to assemble a DNA construct *in vitro*. In some embodiments,

- 38 -

the DNA construct is a non-plasmid construct, while in other embodiments it is incorporated into a vector (e.g., a plasmid). In some embodiments, circular plasmids are used. In preferred embodiments, circular plasmids are designed to use an appropriate restriction enzyme (*i.e.*, one that does not disrupt the DNA construct). Thus, linear

5 plasmids find use in the present invention (See, Figure 1). However, other methods are suitable for use in the present invention, as known to those in the art (See e.g., Perego, "Integrational Vectors for Genetic Manipulation in *Bacillus subtilis*," in (Sonenshein et al. (eds.), *Bacillus subtilis and Other Gram-Positive Bacteria*, American Society for Microbiology, Washington, DC [1993]).

10 In some embodiments, the incoming sequence includes a selective marker. In some preferred embodiments, the incoming sequence includes a chromosomal gene selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *phrC*, *sigB*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD* or fragments of any of these genes (alone or in combination). In
15 additional embodiments, the incoming sequence includes a homologous *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *phrC*, *sigB*, *rapA*, *CssS trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and/or *rocD* gene sequence. A homologous sequence is a nucleic acid sequence having at least 99%, 98%, 97%, 96%, 95%, 94% 93%, 92%, 91%, 90%, 88%, 85% or 80% sequence identity to a *sbo*, *slr*, *ybcO*,
20 *csn*, *spolISA*, *phrC*, *sigB*, *rapA*, *CssS trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and/or *rocD* gene or gene fragment thereof, which may be included in the incoming sequence. In preferred embodiments, the incoming sequence comprising a homologous sequence comprises at least 95% sequence identity to a *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *phrC*, *sigB*, *rapA*, *CssS trpA*, *trpB*,
25 *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, or *rocD* gene or gene fragment of any of these genes. In yet other embodiments, the incoming sequence comprises a selective marker flanked on the 5' and 3' ends with a fragment of the gene sequence. In some embodiments, when the DNA construct comprising the selective marker and gene, gene fragment or homologous sequence
30 thereto is transformed into a host cell, the location of the selective marker renders the gene non-functional for its intended purpose. In some embodiments, the incoming sequence comprises the selective marker located in the promoter region of the gene. In other embodiments, the incoming sequence comprises the selective marker located after the promoter region of gene. In yet other embodiments, the incoming sequence
35 comprises the selective marker located in the coding region of the gene. In further

- 39 -

embodiments, the incoming sequence comprises a selective marker flanked by a homology box on both ends. In still further embodiments, the incoming sequence includes a sequence that interrupts the transcription and/or translation of the coding sequence. In yet additional embodiments, the DNA construct includes restriction sites engineered at the 5 upstream and downstream ends of the construct.

Whether the DNA construct is incorporated into a vector or used without the presence of plasmid DNA, it is used to transform microorganisms. It is contemplated that any suitable method for transformation will find use with the present invention. In preferred embodiments, at least one copy of the DNA construct is integrated into the host 10 *Bacillus* chromosome. In some embodiments, one or more DNA constructs of the invention are used to transform host cells. For example, one DNA construct may be used to inactivate a *sir* gene and another construct may be used to inactivate a *phrC* gene. Of course, additional combinations are contemplated and provided by the present invention.

In some preferred embodiments, the DNA construct also includes a polynucleotide 15 encoding a protein of interest. In some of these preferred embodiments, the DNA construct also includes a constitutive or inducible promoter that is operably linked to the sequence encoding the protein of interest. In some preferred embodiments in which the protein of interest is a protease, the promoter is selected from the group consisting of a *tac* promoter, a β -lactamase promoter, or an *aprE* promoter (DeBoer *et al.*, Proc. Natl. Acad. 20 Sci. USA 80:21-25 [1983]). However, it is not intended that the present invention be limited to any particular promoter, as any suitable promoter known to those in the art finds use with the present invention. Nonetheless, in particularly preferred embodiments, the promoter is the *B. subtilis* *aprE* promoter.

Various methods are known for the transformation of *Bacillus* species. Indeed, 25 methods for altering the chromosome of *Bacillus* involving plasmid constructs and transformation of the plasmids into *E. coli* are well known. In most methods, plasmids are subsequently isolated from *E. coli* and transformed into *Bacillus*. However, it is not essential to use such intervening microorganisms such as *E. coli*, and in some preferred embodiments, the DNA construct is directly transformed into a competent *Bacillus* host.

In some embodiments, the well-known *Bacillus subtilis* strain 168 finds use in the present invention. Indeed, the genome of this strain has been well-characterized (See, Kunst *et al.*, Nature 390:249-256 [1997]; and Henner *et al.*, Microbiol. Rev., 44:57-82 [1980]). The genome is comprised of one 4215 kb chromosome. While the coordinates used herein refer to the 168 strain, the invention encompasses analogous sequences from 35 *Bacillus* strains.

- 40 -

In some embodiments, the incoming chromosomal sequence includes one or more genes selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spollSA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD* gene fragments thereof and homologous sequences thereto.

- 5 The DNA coding sequences of these genes from *B. subtilis* 168 are provided in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 37, SEQ ID NO: 25, SEQ ID NO: 21, SEQ ID NO: 50, SEQ ID NO: 29, SEQ ID NO: 23, SEQ ID NO: 27, SEQ 10 ID NO: 19, SEQ ID NO: 31, SEQ ID NO: 48, SEQ ID NO: 46, SEQ ID NO: 35, and SEQ ID NO: 33.

As mentioned above, in some embodiments, the incoming sequence which comprises a *sbo*, *slr*, *ybcO*, *csn*, *spollSA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh*, *kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD* gene, a gene fragment thereof, or a homologous sequence thereto includes the coding region and may further include immediate chromosomal coding region flanking sequences. In some embodiments the coding region flanking sequences include a range of about 1 bp to 2500 bp; about 1 bp to 1500 bp, about 1 bp to 1000 bp, about 1 bp to 500 bp, and 1 bp to 250 bp. The number of nucleic acid sequences comprising the coding 15 region flanking sequence may be different on each end of the gene coding sequence. For example, in some embodiments, the 5' end of the coding sequence includes less than 25 bp and the 3' end of the coding sequence includes more than 100 bp. Sequences of these genes and gene products are provided below. The numbering used herein is that used in subtilist (See e.g., Moszer et al., Microbiol., 141:261-268 [1995]).

25

The *sbo* coding sequence of *B. subtilis* 168 is shown below:

ATGAAAAAAAGCTGTCATTGTAGAAAACAAAGGTTGTGCAACATGCTCGATCGGAGCCG
CTTGTCTAGTGGACGGTCCTATCCCTGATTITGAAATTGCCGGTGCAACAGGTCTATT
GGTCTATGGGGG (SEQ ID NO:1).

30

The deduced amino acid sequence for Sbo is:

MKKAVIVENKGATCSIGAACLVDGPIPDEIAGATGLFGLWG (SEQ ID NO: 2).

In one embodiment, the gene region found at about 3834868 to 3835219 bp of the 35 *B. subtilis* 168 chromosome was deleted using the present invention. The *sbo* coding region found at about 3835081 to 3835209 produces subtilisin A, an antimicrobial that has

- 41 -

activity against some Gram-positive bacteria. (Søe, Zheng *et al.*, *J. Bacteriol.*, 181:7346–7355 [1994]).

The *s/r* coding sequence of *B. subtilis* 168 is shown below:

- 5 ATGATTGGAAGAATTATCCGTTGTACCGTAAAAGAAAAGGCTATTCTATTAATCAGCTG
GCTGTTGAGTCAGGCCTATCCAATCCTATTAAAGCAAGATTGAAAGAGGCGTTCACAC
GAATCCGTCGTTCAATTTAAAAAGTTCTGCCACACTGGAAGTTGAATTAAACAGA
ATTATTTGACGCAGAACAAATGATGTATGAAAAAAATCAGCGGCGGTGAAGAAGAATGGC
GCGTACATTTAGTGCAAGCCGTACAAGCCGGATGGAAAAGGAAGAATTGTTCACTTT
- 10 ACGAACAGACTCAAGAACAGAACAGCCTGAAACTGCCTCTTACCGCAACCGCAAACGTGA
CGGAATCCAATATAGAAGAATGGAAAGCGCTGATGGCGGAGGCAAGAGAAATCGGCTT
GTCTGTCCATGAAGTCAAATCCTTTAAAAACAAAGGGAAGA (SEQ ID NO:3).

The deduced amino acid sequence for *S/r* is:

- 15 MIGRIIRLYRKRGYSINQLAVESGVSKYLSKIERGVHTNPSVQFLKKVSATLEVELTELF
DAETMMYEKISGGEEEWRVHLVQAVQAGMEKEELFTFTNRLKKEQPETASYRNRKLTES
NIEEWKALMAEAREIGLSVHEVKSFLKTGR (SEQ ID NO: 4).

- 20 In one embodiment, the sequence found at about 3529014 – 3529603 bp of the *B. subtilis* 168 chromosome was deleted using the present invention. The *s/r* coding sequence is found at about 3529131 to 3529586 of the chromosome.

The *phrC* coding sequence of *B. subtilis* 168 is provided below:

- 25 ATGAAATTGAAATCTAAGTTGTTATTGTTGGCCGCAGCCGCAGTTTACAGCG
GCTGGCGTTCTGCTAATGCGGAAGCACTCGACTTCACTGTGACAGAAAGAGGAATGA
CG (SEQ ID NO :13).

The deduced amino acid sequence for *PhrC* is:

- 30 MKLKSCLFVICLAAAIFTAAGVSANAEALDFHVTERGMT (SEQ ID NO: 14)

Additionally, the coding region found at about 429531 to 429650 bp of the *B. subtilis* 168 chromosome was inactivated by an insertion of a selective marker at 429591 of the coding sequence.

The *sigB* coding sequence of *B. subtilis* 168 is shown below:

- 35 TTGATCATGACACAAACCATAAAAACATACGAAACTAACTAAAGATGAAGTCGATCGGCT
CATAGCGATTACCAAACAAAGCAAGATGAACAAGCGCAGGAAACGCTTGTGCGGGTG
TATACAAATCTGGTTGACATGCTTGCAGAAAAAAACTCAAAAGGCAAAAGCTTCCACGA
GGATCTCCGCCAGGTGGCATGATCGGGCTGCTAGGGCGATTAAGCGATACGATCC
TGTTGTGGCAAATCGTTGAAGCTTTGCAATCCGACAATCATCGGTGAAATTAAAC
- 40 GTTTCCTCAGAGATAAAACATGGAGCGTTCATGTGCCAGACGAATTAAAGAACTCGGT
CCAAGAATCAAATGGCGGTTGATCAGCTGACCACTGAAACACAAAGATCGCCGAAAG
TCGAAGAGATTGCCAATTCTCGATGTTCTGAAGAAGAGGTTCTGAAACGATGGAA
ATGGGAAAAGCTATCAAGCCTTATCGTTGACCACAGCATTGAAGCGGATTGGACG
GAAGCACTGTCACGATTCTGATATCGTCGGATCACAGGAGGACGGATATGAGCGGGT

- 42 -

CAACCAGCAATTGATGCTGCAAAGCGTGCTTATGTCCTTCAGACCGTGAGAAACAAA
 TCATAGACCTTACGTATATTCAAAACAAAAGCCAAAAGAAACTGGGGACATTCTCGGT
 ATATCTCAAATGCACGTCTCGCGCTGCAACGCAAAGCTGTGAAGAAGCTCAGAGAGG
 CCTTGATTGAAGATCCCTCGATGGAGTTAATG (SEQ ID NO:9).

5

The deduced amino acid sequence for SigB is:

MIMTQPSKTTKLKDEVDRLLSDYQTQKQDEQAQETLVRVYTNLVDMILAKKYSKGKSFHD
 LRQVGMIGLLGAIKRYDPVVGKSFEAFIAPITIIGEIKRFLRDKTWSVHPPRIKELGPRIKMA
 VDQLTTETQRSPKVEEIAEFLDVSEEEVLETMEMGKSYQALSVDHSIEADSDGSTVTILD
 10 VGSQEDGYERVNQQMLQSVLHVLSREKQIIDLTYIQNKSQKETGDILGISQMHSRLQ
 RKAVKKLREALIEDPSMELM (SEQ ID NO: 10).

Additionally, the coding sequence is found at about 522417 to 5232085 bp of the *B. subtilis* 168 chromosome.

15

The *spolISA* coding sequence of *B. subtilis* 168 is shown below:

ATGGTTTTATTCTTCAGATCATGGTCTGGTGCATCGTGGCCGGACTGGGGTTATACGT
 GTATGCCACGTGGCGTTCGAACAGCGAAGGTCAAAGAAAAATGTCCGCCATTGGAAA
 ACTTGGTATTGCTTTGCTGGCGCTATGGTACTGGACATATGAGCCCACCTC
 CCTATTTACCCACTGGAACGGTATCTCATTGTCGAGTCAGTTTGCTTGATTGATG
 20 CTTTTATCTTCTTAAGTCATATGCAAAACTGGCCGGCAGCGAGCTTGAAACAGAC
 ACAAGAGAAATTCTGAAGAAAACAACGAAATGCTCCACATGTATCTCAATCGGCTGAA
 AACATACCAATACCTATTGAAAAACGAACCGATCCATGTTATTATGGAAGTATAGATGC
 TTATGCTGAAGGTATTGATAAGCTGCTGAAAACCTATGCTGATAAAATGAACCTAACGG
 CTTCTTTGCCACTATTCGACACAGGCTGATAAAAGACCGGTTAACCGAGCATATGGAT
 25 GATCCGGCAGATGTACAAACACGGCTCGATCGAAAGGATGTTATTACGACCAATACG
 GAAAAGTGGTCTCATCCCTTTACCATCGAGACACAGAACTATGTCATCAAGCTGACG
 TCTGACAGCATTGTCACGGATTGATTATGCTATTACGTCTAACGAGCATATAT
 GATTGGTGCTGCCATTGAGGAGGAAGGTGAAGGA (SEQ ID NO:11).

30

The deduced amino acid sequence for SpolISA is:

MVLFFQIMWCIVAGLGLYYATWRFEAKVKEKMSAIRKTWYLLFVLGAMVYWTYEPTSL
 FTHWERYLIVAVSFALIDAFIFLSAYVKKLAGSELETDREILEENNEMLHMYLNRLKTYQY
 LLKNEPIHVYYGSIDAYAEGIDKLLKTYADKMNLTLASLCHYSTQADKDRLTEHMDDPADV
 35 QTRLDRKDYYDQYGKVLIPFTIETQNYVIKLTSDSIVTEFDYLLFTSLTSIYDLVPIEEE
 EG (SEQ ID NO: 12).

Additionally, the coding region is found at about 1347587 to 1348714 bp of the *B. subtilis* 168 chromosome.

40

The *csn* coding sequence of *B. subtilis* 168 is shown below:

ATGAAAATCACTATGCAAAAGCAGATTGGAAAAAGCAGCGATCTCATTACTTGT
 TTCACCATTTTACCTGATGATGAGCGAACAGGTTTGCAGGGACTGAATAA
 AGATCAAAAGCGCCGGCGAACAGCTGACAAGTATCTTGAAAACGGCACACGGA
 45 GATCCAATATGGATATGTAGAGCGATTGGATGACGGCGAGGCTATACATGCGGACGG
 GCAGGCTTACAACGGCTACCGGGATGCATTGGAAGTAGTGGAAAGTATACACAAAGG
 CAGTTCCGAATAACAAACTGAAAAGTATCTGCCTGAATTGCGCCGCTGGCCAAGGA
 AGAAAGCGATGATACAAGCAATCTCAAGGGATCGCTCTGCCTGGAAGTCGCTTGCA
 AATGATAAGGAATTGCGCCGCTCAAGACAAAGTAAATGACCATTGTATTACGCC

- 43 -

TGCCATGAAACGATCGGATAATGCCGGACTAAAAACAGCATTGGCAAGAGCTGTGATG
TACGATACGGTTATTCAAGCATGGCGATGGTGTGACCTTGACTCTTTATGCCTTGAT
TAAACGTACGAACAAAAAGCGGGCGGATCACCTAAAGACGGAATAGACGAGAAGAAG
TGGTTGAATAAATTCTGGACGTACGCTATGACGATCTGATGAATCCGGCCAATCATGA
5 CACCCGTGACGAATGGAGAGAACATAGCTTGGCCGTGTGGACGTGCTCGCTATCGCC
AAGGAGAACAACTATAATCTAACGGACCGATTATGTTGTTCAAACGAGTACGGTAA
TTTGTAATCAA (SEQ ID NO:7).

The deduced amino acid sequence for Csn is:

10 MKISMQKADFWKKAISLLVFTMFTLMMSETVFAAGLNKDQKRRAEQLTSIFENGTEIQ
YGYVERLDDGRGYTCGRAGFTTATGDALEVVVEVTAKAVPNNKLKKYLPELRRLAKEESD
DTSNLKGFASAWKSILANDKEFRAAQDKVNDHLYQPAMKRSNDAGLKTALARAVMYDT
VIQHGDGDDPDPSFYALIKRTNKKAGGSPKDGIKEKKWLNKFLDVRYDDLMPANHDTRD
EWRESVARVDVLRSIAKENNYNLNGPIHRSNEYGNFVIK (SEQ ID NO: 8).

15

Additionally, the coding region is found at about 2747213 to 2748043 bp of the *B. subtilis* 168 chromosome.

The *ybcO* coding sequence of *B. subtilis* 168 is shown below:

20 ATGAAAAGAAACAAAAAGAATGGGAATCTGTGAGTAAAAAAGGACTTATGAAGCCGG
25 GAGGTACTTCGATTGTGAAAGCTGCTGGCTGCATGGCTGTTGGCCTCGAAGAGTAT
TGCTATGACACGTGTTGTGACTTCCGCATCCTGCTATGAGAGCTATT (SEQ ID NO:5).

The deduced amino acid sequence for *YbcO* is:

MKRNQKEWESVSKGLMKPGGTSIVKAAGCMGCWASKSIAMTRVCALPHPAMRAI
25 (SEQ ID NO: 6).

Additionally, the coding region is found at about 213926 to 214090 bp of the *B. subtilis* 168 chromosome.

The *rapA* coding sequence of *B. subtilis* 168 is shown below:

30 TTGAGGATGAAGCAGACGATTCCGCTCTTATGTCGGGCTTAAATTAAATGAATGGTA
TACTCATATCCGGCAGTTCCACGTCGCTGAAGCCGAACGGGTCAAGCTCGAAGTAGAA
AGAGAAATTGAGGATATGAAAGAACCAAGATTGCTGCTGTATTATTCTTAATGGA
GTTCAGGCACCGTGTATGCTGGATTACATTAAGCCTTTGGAGAGGACACGTCGCAG
CTAGAGTTTCAGAATTGTTAGAAGACATCGAAGGGAATCAGTACAAGCTGACAGGGCT
35 TCTCGAATATTACTTAATTTCGAGGAATGTATGAATTAAAGCAGAAGATGTTGTC
AGTGCCATGATGTATTATAAACGGGCAGAAAAGAATCTTGCCTCGTCGGATGATAT
TGAGAAAGCAGAGTTGCTTTAAATGGCTGAGATTTCACAATTAAACAAACCTA
TGTTTCGATGAGCTACGCCGTTCAGGCATTAGAAACATACCAAATGTATGAAACGTACA
CCGTCGCAGAATCCAATGTGAATTGTTATTGCAAGGTAATTATGATGATATGCAGTAT
40 CCAGAAAGAGCATTGCCCACTTAGAAGTGGCTTATGATCTTGCAGAAGAAAGAAGGCA
ATCCCCGCCTGATCAGTTGCGCTATATAATCTCGGAAACTGCTATGAGAAAATGGGT
GAACTGCAAAGGGAGCCGAATACTTGGGAAATCTGTTCTATTGCAAGTCGGAAAA
GTTCGATAATCTCCGCATTCTACTCTTAAACACAAGTTCTGTATAAACAAAAAAAT
GACGCCGAAGCGAAAAAGTATCGTGAAGGATTGGAAATGCCCGTCAATACAGTG
45 ATGAATTATTGTTGGAGCTTTCAATTTCACATGCGTTATACGGAAAAACATTGACA
CAGAATCAGTCTCACACACCTTCAATTCTGAAGAACATATGCTGTATCCTTATATTG
AAGAGCTGGCGCATGATGCTGCCAATTCTATAGAAAACGGACAGCCGAAAAAGC

- 44 -

ACTTCATTTATGAGAAAATGGTGCACGCACAAAAACAAATCCAGAGAGGAGATTGTT
TATATGAAATC (SEQ ID NO :15).

The deduced amino acid sequence for RapA is:

5 MRMKQTIPSSYVGLKINEWYTHIRQFHVAEAERVKLEVEREIEDMEEDQDLLLYYSLMEF
RHRVMLDYIKPFGEDTSQLEFSELLEDIEGNQYKLTGLLEYYYNFFRGMYEFKQKMFVSA
MMYYKRAEKNLALVSDDIEKAFAFKMAEIFYNLKQTYVSMSYAVQALETYQMYETYTVR
RIQCEFVIAGNYDDMMQYPERALPHLEALDLAKKEGNPRLISSALYNLGNCYEKMGELQK
AAEYFGKSICKSEKFNDLPHSIYSLTQVLYKQKNDAEAQKKYREGLEIARQYSDELFVE
10 LFQFLHALYGKNIDTESVSHTFQFLEEHMLYPYIEELAHDAAQFYIENGQPEKALSFYEK
VHAQKQIQRGDCLYEI (SEQ ID NO: 16)

Additionally, the coding region is found at about 1315179 to 1316312 bp of the *B. subtilis* 168 chromosome.

15 The *Css* coding sequence of *B. subtilis* 168 is shown below:

ATGAAAAACAAGCCGCTCGCGTTTCAGATATGGGTTGTATATCCGGCATCCTGTTAG
CGATATCGATTTACTGCTTGTATTTCAAACACGCTGCGAGATTTTCACTAAT
GAAACGTATACGACGATTGAAAATGAGCAGCATGTTCTGACAGAGTACCCGCTGCCA
GGTCGATTGAAAGGCCTTACAGCGAGGAAGCGACGGCGCCGACAACACTGTCCG
20 CTCCGTACAGCACGTGCTCCTTCCTGAAAATGAAGAGGCTTCTCAGACAAGGATTAA
AGCATTCTGTATCTTCAATTATCCACAAGGTGTACAAGCTGGCTGATAAGCAGGAAG
CTAAAAAGAACGTTACAGCGCCGACGTCAATGGAGAGAAAGTGTGTTTCATTAA
AAAGGGACTTCCGTCATGGACAATCAGCGATGATGCTCTTACCGCCTTGATTCT
TATCGGGACGATTGGCCTATACCTGTTCAAACAGCTTCTGTTTATTATAGCTGTCGT
25 CATTTTATTAAGCTGGATTCCGGCTATTGGCTTGCAAAGTATTATCAAGGCCTTGT
TATCATTGAAAAACACGTCAAACGGATTCTGAACAGGATTGGGATGACCCAGTAAA
AGTGGACCGGAAAGATGAAATCGGCAAATTGGGCCATACCATCGAAGAGATGCGCC
AAAAGCTTGTGCAAAAGGATGAAACAGAAAGAACTCTATTGCAAATATCTCATGA
TTTAAAAACGCCGGTATGGTCATCAGAGGCTATACACAATCAATTAAAGACGGGATT
30 TTCCCTAAAGGAGACCTTGAAAACACTGTAGATGTTATTGAATGCGAAGCTTAAAC
TGGAGAAAAAAATAAGGATTATTATTTAACGAAGCTGGATTATTAGCGAACCAA
AAAGTGCAGCACGACATGTTCACTATTGTGGAAAGTGACAGAAGATCATCGAACGA
TTGAAGTGGCGCGGAAAGAACTATCGTGGAAATTGATGTAGAAGAGGATATTG
35 ATGCCGGCGATCCGGAGCAATGGAACAAACTCCTCGAAAACATTGGAAAATCAA
ATCCGCTATGCTGAGACAAAAATAGAAATCAGCATGAAACAAAGATGATCGAAATATCG
TGATCACCATTAAGGAGACCTTGCAATTGAGATGAGATGCTCTCCAGCCTCTA
TGAGCCTTTAATAAAAGGGAGAAAGGGCAATTGGCATTGGCTAAGCATCGAAAA
CGAATTAACTCTCATAAGGCATCTCAATTGAAAATGACAAAACGGGTGTATC
40 ATACCGCATAGCAGTGCCAAA (SEQ ID NO:17).

40

The deduced amino acid sequence for *Css* (GenBank Accession No. O32193) is:

MKNKPLAFQI WVVISGILLAISILLVLFSNTLRDFFTNETYTTIENEQHVLTEYRLPGSIE
RRYYSEEATAPTVRSVQ HVLLPENEASSDKDLSILS SSFIHKVYKLADKQEAKKKR
YSADVNGEKVFFVIKKGLSVNGQSAMMLSYALDSYRDDLAYTLFKQLLFIIAVVILLSWIPAI
45 WLAKYLSRPLVSFEKHVKRISEQDWDDPVKVDKRDEIGKLGHTEEMRQKLVQKDETER
TLLQNISHDLKTPVMVIRGYTQSICKDGFIPKGDLENTVDVIECEALKLEKKIKDLLYLTKL
LAKQKVQHDMFSIVEVTEEVIERLKWARKEELSWEIVEEDILMPGDPEQWNKLENILENQI
RYAETKIEISMKQDDRNIITIKNDGPHIEDEMLSSLYEFPNKGKGEFGIGLSIVKRILTLHK
ASISIENDKTGVSYRIAVPK (SEQ ID NO:18).

- 45 -

Additionally, the gene region is found at about 3384612 to 3386774 bp of the *B. subtilis* 168 chromosome.

The *fbp* coding sequence of the Fbp protein (fructose-1,6-biophosphatase) of *B. subtilis* 168 is shown below:

```

ATGTTAAAATAATGTCATACTTTAAATTACCTTATCATGCACATGCTCATAAAGA
GGGGTTTATTCTAAAAGGGGATGGACGGTTGGAAAGCAAGTACCTAGATCTACT
CGCACAAAAATACGATTGTGAAGAAAAAGTGGTAACAGAAATCATCAATTGAAAGCG
ATATTGAACCTGCCAAAAGGCACCGAGCATTGTCAGTGATCTGCACGGAGAGTAT
10 CAGGCATTCCAGCACGTGTCAGCAATGGTCAGGACGAGTCAAAGAGAAGATACG
CGACATCTTCAGCGGTGTCATTACGATAGAGAAATTGATGAATTAGCAGCATTGGTC
TATTATCCGGAAGACAAACTGAAATTAAATCAAACATGACTTGTGCGAAAGAAGCGT
TAAACGAGTGGTATAAAGAAACGATTCACTGAATGATTAAGCTCGTTCATATTGCTC
CTCTAAGTATACCGCTCAAATTACGCAAAGCACTGCCTGCCAATTGCTTATATT
15 ACGGAGGGAGCTGTTATACAAAACAGAACAAAGCTGGCAACAAGGAGCAATTACTCC
GAAATCATTGATCAGATCATTGAACTTGGCCAAGGCCATAAGCTGATCACCGGCCTT
GCTTACAGCGTTCAGCGATTGGTGGTCGACCCTGCATGTGGTGGCGATATTAT
GACCGCGGCCCGCAGCCGGATAGAATTATGGAAGAACTGATCAACTATCATTCTGTC
GATATTCACTGGGAAATCACGATGTCCTTGGATCGGCGCTATTCCGGTTCCAAA
20 GTGTGCCTGGCCAATATTATCCGCATCTGTGCCCCTACGACAACCTGGATATTATG
AGGACGTGTACGGCATCAACCTGAGACCGCTGCTGAACCTGGCCGAAAATATTATG
ATGATAATCCAGCGTCCGTCAAAAGCAGACGAAAACAGG
CCAGAGGATGAGATTAAGCAAATCACAAAATCCATCAAGCATTGCCATGATCCAAT
TCAAGCCTGAGAGCCGATTATCAAGAGACGGCGAACTTTAATATGGAAGAGCGGC
25 TGTTATTAGAGAAAATAGACTATGACAAAAATGAAATCACGCTGAACGGAAAACATA
TCAACTGGAAAACACCTGCTTGCACGATTAAATCCGGAGCAGCCAGATCAGCTATT
AGAAGAAGAAGCAGAAGTCATAGACAAGCAGCTGCTATTCTCTGTCCAGCATTCCGAAA
GCTGGGCCCATATGAATTGATGAAAAAAGGCAGCCTTATTAAAATATAAC
GGCAACCTGTTGATTCACGGCTGTATTCCAGTTGATGAAAACGGCAATATGGAACG
30 ATGATGATTGAGGATAAACCGTATGCGGGCCGTGAGCCTGCTCGATGTATTGAAACGA
TTCTTGCAGGAAAGCCTTGCACCCCGAAGAAACCGATGACCTGGCGACAGATATG
GCTTGGTATTGACAGGCGAATACTCCTCCCTCTCGGAAAACGCCATGACG
ACATTGAGCGCTATTTCATCAAAGAGAAGGAAACGCATAAAGAGAAGAAAACCGT
ATTATTATTACGAGAAGACGAGGCAACCTGCCAAACATCCTGGCAGAATTGGCC
35 TCAATCCAGATCACGGCCATATCATCAAACGCCATACACCTGTTAAAAGAAAATCGAAG
GAGAAGACCCAAATCAAAGCAAACGGAAAAATGATCGTCATCGACGGCGCTTCTCCA
AAGCCTACCAATCCACAACAGGCATCGCCGGCTACACGCTGCTATAACACTCCTACG
GCATGCAGCTCGTCGCCATAAACACTTCAATTCAAGGCAGAAGTCTAAGCACCG
GAACCGACGTCTAACGGTCAAACGATTAGTGGACAAAGAGCTTGAGCGGAAAGAAG
40 TGAAGGAAACGAATGTGGGTGAGGAATTGTTGAGGAAGTTGCGATTGCGATTAGAGAGTT
TGCAGGAGTATCGGTATATGAAG (SEQ ID NO:19).

```

The deduced amino acid sequence of the Fbp protein is:

```

MFKNVILLNSPYHAHAKEGFILKRGWTVLESKYLDLLAQKYDCEEKVVTEINLKAILNL
45 PKGTEHFVSDLHGEYQAFQHVLRNGSGRVKEKIRDIFSGVIYDREIDEALVYPED
KLKLIKHDFAKEALNEWYKETIHRMIKLVSYCSSKYTRSKLRKALPAQFAYITEELLYK
TEQAGNKEQYYSEIIDQIIELGQADKLITGLAYSQQLVVDHLHVVGDIYDRGPQPDRIM
EELINYHSVDIQWGNHDVLWIGAYGSKVCLANIIRICARYDNLDIIEDVYGINLRPLLN
LAEKYYDDNPAPRKADENRPEDEIKQITKIHQAIAMIQFKLESPIIKRRPNFNMEERLL

```

- 46 -

LEKIDYDKNEITLNGKTYQLENTCFATINPEQPDQLLEEEAEVIDKLLFSVQHSEKLGRH
 MNFMMKKGSLYLKYNGNLLIHGCIPDENGNMETMMIEDKPYAGRELLDVERFLREAF
 AHPEETDDLATDMAWYLWTGEYSSLFGKRAMTFERYFIKEKETHKEKNPYYLREDE
 5 ATCRNILAEFGLNPDHGHIINGHTPVKEIEGEDPIKANGKIVIDGGFSKAYQSTTGIAGYT
 LLYNSYGMQLVAHKHFNASKAEVLSTGTDVLTVKRLVDKELERKKVKETNVGEELLQEAI
 LESLREYRYMK (SEQ ID NO:20).

Additionally, the coding region is found at about 4127053 to 4129065 bp of the *B. subtilis* 168 chromosome.

10

The *alsD* coding sequence of the *alsD* protein (alpha-acetolactate decarboxylase) of *B. subtilis* 168 is shown below:

15 ATGAAACGAGAAAGCAACATTCAAGTGCTCAGCCGTGGTAAAAAGATCAGCCTGTG
 AGCCAGATTTCAGTATCAACAATGACTTCTATTAGACGGAGTATGACGGAG
 ATTTGAACGTCAAGAGATTCCGAAATATGGAGACTTCGGTATCGGAACCTTAACAA
 GCTTGACGGAGAGCTGATTGGGTTGACGGCGAATTTACCGTCTCGCTCAGACGG
 AACCGCGACACCGGTCCAAAATGGAGACC GTT CACCGTTCTGTTCATTA CGTTCTT
 20 ACACCGGACATGACGCAACAAATTGATGCGAAAATGACACGCGAAGACTTGA AAAA
 GAGATCAACAGCATGCTGCCAAGCAGAAACTTATTTATGCAATT CGCATTGACGGAT
 TGTTAAAAAGGTGCAGACAAGAACAGTAGAACACTTCAAGAAAACCTTACGTGCCAAT
 GGTTGAAGCGGTCAAACACAGCCGATTTCAACTTCGACAACGTGAGAGGAACGAT
 TGTAGGTTCTTGACACCAGCTTATGCAAACCGGAATCGCCGTTCTGGCTATCACCTG
 25 CACTTCATTGACGAAGGACGCAATT CAGGCGGACACGTTTGACTATGTGCTTGAG
 GATTGCACGGTTACGATTCTCAAAAATGAACATGAATCTCAGACTCCGAACACAG
 CGGATTCTTAATGCGAATCTGGATAACCGT GATT TGCGAAAGATATCGAAACAC
 TGAAGGAAGCCCTGAA (SEQ ID NO:21).

30

The deduced amino acid sequence *AlsD* protein sequence is:

MKRESNIQVLSRGQKDQPVSQIYQVSTMTSLLDGVYDGFELSEIPKYGDFGIGTFNKL
 GELIGFDGEFYRLRSDGTATPVQNGDRSPFCSTFFT PDMTHKIDAKMTREDFEKEINSM
 LPSRNLFYAIRIDGLFKKVQTRTVELQEKPYVPMVEAVKTQPIFNFDNVRGTIVGFLTPAYA
 NGIAVSGYHLHFIDEGRNSGGHVFDYVLEDCTVTISQKMNMNLRLPNTADFFNANLDNP
 35 FAKDIETTEGSPE (SEQ ID NO:22).

Additionally, the coding region is found at about 3707829-3708593 bp of the *B. subtilis* 168 chromosome.

40

The *gapB* coding sequence of the *gapB* protein (glyceraldehyde-3-phosphate dehydrogenase) of *B. subtilis* 168 is shown below:

45

ATGAAGGTAAAAGTAGCGATCAACGGGTTT GGAAGAATCGGAAGAATGGTTTTAGA
 AAAGCGATGTTAGACGATCAAATTCAAGTAGTGGCCATTAACGCCAGCTATTCCGCA
 GAAACGCTGGCTCTTAAATAAAGTATGACACAATT CACGGCAGATACGACAAAGAG
 GTTGTGGCTGGTGAAGATAGCCTGATCGTAAATGGAAAGAAAGTGCTTTGTTAAACAA
 GCCGTGATCCAAAACAGCTGCCCTGGCGGGAAATATGATATTGACATAGTCGTCGAAG
 CAACAGGGAAAGTTAATGCTAAAGATAAAGCGATGGGCCATATAGAAGCAGGTGCAA

- 47 -

AAAAAGTGATTTGACCGCTCCGGAAAAAAATGAAGACGTTACCATTGTGATGGCG
 TAAATGAGGACCAATTGACGCTGAGCGCATGTCATTATTCAAATGCGTCATGCAC
 GACAAATTGCCTTGCGCCCTGTTGAAAAGTGTGGATGAAGAGTTGGCATTGAGAG
 CGGTCTGATGACTACAGTTCATGCGTATACGAATGACCAAAAAAATTGATAACCCG
 5 CACAAAGATTGCGCCGGCGCGGGCTTGCGGTGAATCCATCATTCCAACAACAACA
 GGAGCGGCAAAGGCGCTTCGCTGTGCTGCCGCATCTGAAAGGAAACTCACGG
 CCTCGCCTTGCCTGTCCTGTTCCGAACGTCATTGGTGTCTCGTTGATCTG
 AAAACGGATGTTACGGCTGAAGAAGTAACGAGGCATTAAACGCGCTGCCAAAACG
 TCGATGTACGGTGTACTTGATTACTCAGATGAACCGCTC
 10 GTTTCGACTGATTATAATACGAATCCGCATTAGCGGTCAATTGACGGGCTTACAACAA
 TGGTAATGGAAGACAGGAAAGTAAAGGTGCTGGCGTGGTATGACAACGAATGGGGC
 TACTCCTGCAGAGTTGATCTAACCGCCATGTAGCGGCACGAATGAAACATCCG
 TCTGCTGTA (SEQ ID NO:23).

15 The deduced amino acid sequence of the GapB protein is:

MKVKVAINGFGRIGRMVFRKAMLDDQIQVVAINASYS AETLAHLIKYDTI HGRYDKEVVA
 GEDSLIVNGKKVLLNSRDPKQLPWREYDIDIVVEATGKFNAKDKAMGHIEAGAKKVILT
 APGKNEDVTIVMGVNEDQFDAERHVIISNASCTNCLAPVVVLKGLALRVPVNVS
 20 TNDQKNIDNPBKDLRRARACGESIPTTGAAKALSLVPLHLKGKLHGALRVPVNVS
 DLVVDLKDVTAAEVNEAFKRAAKTSMYGVLDYSDEPLVSTDYNTNPHSAVIDGLTTMVM
 EDRKVVKVLAWYDNEWGYSCRVVDLIRHVAARMKHPSAV (SEQ ID NO:24).

25 Additionally, the coding region is found at about 2966075-2967094bp of the *B. subtilis* 168 chromosome.

The *Kbl* coding sequence of the *Kbl* protein (2-amino-3-ketobutyrate CoA ligase) is shown below:

30 ATGACGAAGGAATTGAGTTTAAAAGCAGAGCTTAATAGTATGAAAGAAAACCATA
 CATGGCAAGACATAAAACAGCTTGAATCTATGCAGGGCCCATCTGTCACAGTGAATC
 ACCAAAAAGTCATTCACTGCTATCTTCAATAATTACCTCGGATTCACTTCACATCCTAGA
 CTCATCAACGCCGCACAGGAGGCCGTTCAGCAGTATGGAGCCGGCACCGGATCAGT
 GAGAACGATTGCGGGTACATTACAATGCATCAAGAGCTTGAGAAAAAGCTGGCAGC
 35 CTTAAAAAAACGGAGGCCGCACTTGTATTCCAATCAGGCTTCACAACAAACCAAGG
 CGTACTTCAAGTATTCTATCAAAAGAGGACATTGTCTCAGATGAATTGAACCAT
 GCCTCTATTATTGACGGAATTGACTGACAAAGGCGATAAAAAGGTGTATCAGCAC
 GTCAATATGAGTGTAGTAGCGGGTGCTGAGAAAGTCATGAATTATCGGATGCGT
 CTGATTGTGACAGACGGCGTATTTCATGGATGGCAACATAGCTCCTGCGTGTATA
 40 TTGTAGAGCTCGCTGAGAAATATGACGCATTGTGATGGTGGATGACGCCATGCAT
 CGCGAGTACTTGGCGAAAACGGCAGGGAACGGTGAATCACTCGGTCTTGACGGC
 AGAGTGCATATTCAAGGTGCGAACATTAAGCAAGGCAATCGGAGTGCTCGGCGGCTA
 CGCTGCAGGTTCAAAGGTGCTGATCGATTATTGCGCCATAAAGGCCGTCCATTGTTA
 TTCAGCACATCTCATCCGCCGGCAGTCACTGCAGCTGTATGGAAGCGATTGTC
 45 TTGCTTGAAGAGGCCGGAGCATATGGAGCGCTTGTGGGAGAATACTGCCATTAA
 GCAATGCTTGTGAAAATGGGTCTGACTCTCACGAAGAGTGAACACGCCGATTCTTCT
 ATTAAATAGGTGATGAAGGTGTGGCAAAGCAATTTCAGATCAGCTCCTTCTCGCG
 GTGTTTTGCCAAAGTATCGTTTCCGACTGTAGCAAAGGGAAAAGCCAGAAATCG
 CACGATTATAACAGCAGAGCACACCAAAGATGAACGGATCAGGCCTTGATGTCAT
 50 CGAAAAGACGGCAAAGGAGCTCCAGCTATTG (SEQ ID NO:25).

- 48 -

The deduced amino acid sequence of the Kbl protein is:

5 MTKEFEFLKAELNSMKENHTWQDIKQLESMQGPSVTVNHQKVQLSSNNYLGFTSHPRLI
 NAAQEAVQQYGAAGTGSVRTIAGTFTMHQELEKKLAFFKTEAALVFQSGFTTNQGVLSSI
 LSKEDIVISDELNHASIIDGIRLTAKDKVYQHVNMSDLERVLRKSMNYRMRLIVTDGVFS
 MDGNIAPLPDIVE LAEKYDAFVMVDDAHASGVLGENGRTVNHFGLDGRVHIQVGTLSK
 AIGVLGGYAAGSKVLDYLRLHKGPFLFSTSHPPAVTAACMEAIDVLLEEPEHMERLWEN
 TAYFKAMILVKMGLTLKSETPILPILIGDEGVAKQFSDQLLSRGVFAQSIVFPTVAKGKARI
 RTIITAEHTKDELDQALDVIEKTAKELQLL (SEQ ID NO:26).

10 Additionally, the coding region is found at about 1770787 – 1771962 bp of the *B. subtilis* 168 chromosome.

The *PckA* coding sequence of the *PckA* (phosphoenolpyruvate carboxykinase) of *B. subtilis* 168 is shown below:

15 ATGAACTCAGTTGATTGACCGCTGATTACAAGCCTTATTAACATGTCCAAATGTGC
 GTCATAATTATCAGCAGCACAGCTAACAGAAAAAGTCTCTCCCAGAACGAAGGCAT
 TTTAACATCCACAGGTGCTGTTCGCGCGACAACAGGGCGCTTACACAGGACGCTCACC
 TAAAGATAAAATTCATCGTGAGGAAGAAAGCACGAAAAATAAGATCGATTGGGGCC
 GGTGAATCAGCCGATTTAGAAGAAGCGTTGAGCGGCTGTACACGAAAGTTGTCAG
 20 CTATTAAAGGAGCAGATGAACTGTTGTTTCGAAGGATTGCGATGGCACAATTATTGCG
 GAAATACAGGCTGCCGATCACTGTCGTAATGAGTTGCGATGGCACAATTATTGCG
 CGGCAGCTGTTATCCGTCGGAAAGGAAATGATAAGAAAACAGTTGAGCAGCCGTT
 ACCATTCTTCTGCTCCGCAATTCAAAGCGGATCCAACAGACGGCACTCATTCCG
 AAACGTTTATTATTGTCCTTCGAAAAGCGGACAATTAACTCGGCGGAAGTGGAGTA
 25 TGCCGGTGAAATGAAGAAGTCCATTTCCTCCATTATGAATTTCCTGTCGCTGAAAGA
 GATATTTCATCTATGCACTGCTCCGCCAATGTCGGTAAAAAGGGCATGTCGCCCTT
 TCTTCGGACTGTCAGGAACAGGAAAGACCACCCCTGTCGGCAGATGCTGACCGCAAG
 CTGATCGGTGACGGATGAACATGGCTGGTCTGATAACAGGCGTCTTAATATTGAAGGC
 GGATGCTACGCTAACGTGATTCAATTAAAGCGAGGAAAAGGAGGCCGAAATCTTAAC
 30 GCGATCCGCTTCGGGTCTGTTCTGAAAATGTCGTTGGATGAAGATAACCGCGAA
 GCCAATTATGATGATTCTCTATACTGAAAACACGCCGGCAGCTACCCGATTCTATA
 TGATTAATAACATCGTGAECTCAAGCATGGCCGGCATCCGTCAGCATTGTATT
 GACGGCTGATGCCCTCGGAGTCTGCGCCGATCAGCAAACTAACGAAGGAGCAGG
 TGATGTACCATTTTGAGCGGTTACAGAGTAAGCTGCCGGAACCGAACGTGGTG
 35 TCACGTCTCTGAAACGACGTTTCTACATGCTTCGGCTCACCGTCTGCCGCTTCC
 TGCTCACGTCTATGCTGAAATGCTCGGAAAAAGATCGATGAACACGGCGCAGACGT
 TTTCTTAGTCATACCGGATGGACCGGGGGCGCTACGGCACAGGCGAACGAATGA
 AGCTTTCTTACACTAGAGCAATGGCAAAGCAGCGATTGAAGGCAAATTAGAGGATG
 CTGAAATGATAACTGACGATAATTTCGGCTGCACATTCCGGCCATGTTCTGGCGT
 40 TCCTGATCATATCCTCAGCCTGAAAACACGTGGACCAACAAGGAAGAATACAAAGAA
 AAAGCAGTCTACCTTGCAAATGAATTCAAAGAGAACTTAAAAGTTCGCACATACCG
 ATGCCATCGCCCAGGCAGGCAGGCCCTCGTA (SEQ ID NO:27).

The deduced amino acid sequence of the *PckA* protein is:

45 MNSVDLTADLQALLTCPNVRHNLSAALTEKVLSRNEGILTSTGAVRATTGAYTGRSPKD
 KFIVEEESTKNKIDWGPVNQPISEEAFERLYTKVVSYLRKDERELFVFEFGAGADEKYRLPI
 TVVNEFAWHNLFARQLFIRPEGNDKKTVEQPFTILSAPHFKADPKTDGTHSETFIIVSF
 EKRTILIGGTEYAGEMKKSIIFSIMNFLPERDILSMHCSANVGEKGDVALFFGLSGTGKT
 50 TLSADADRKLIGDDEHGWSDTGVFNIEGGCYAKCIHLSEEKEPQIFNAIRFGSVLENVV

- 49 -

DEDTREANYDDSFYTENTRAAYPIHMINNIVTPSMAGHPSAIVFLTADAFGLVLPPISKLT
KEQVMYHFLSGYTSKLAGTERGVTSPEFTSTCFGSPFLPLPAHVYAEMLGKKIDEHGAD
VFLVNTGWTGGYGTGERMKLSYRAMVKAIAEGKLEDAEMITDDIFGLHIPAHVPGVPD
HILQPENTWTNKEEYKEKAVYLANEFKENFKFAHTDAIAQAGGPLV (SEQ ID NO:28).

5

Additionally, the coding region is found at about 3128579-3130159 bp of the *B. subtilis* 168 chromosome.

The *prpC* coding sequence of the *prpC* protein (protein phosphatase) of *B. subtilis* 168 is shown below:

10

TTGTTAACAGCCTTAAAAACAGATACAGGAAAAATCCGCCAGCATAATGAAGATGATG
CGGGGATATTCAAGGGAAAAGATGAATTATATTAGCGGTTGTCGCTGATGGCATGG
GCGGCCATCTGCTGGAGATGTTGCGAGCAAGATGGCTGTGAAAGCCATGGGGAG
AAATGGAATGAAGCAGAGACGATTCCAAC TGCGCCCTCGGAATGTGAAAATGGCTC
15 ATTGAACAGATTCTATCGTAAACAGCAAATATACGATCACGCTCAAGCCCACGAAG
AATGCCAAGGCATGGGACGACGATTGTATGTGACTTTACGGGGAAAACGGTT
CTGTTGCCCATATCGGAGACAGCAGATGCTATTGCTTCAGGACGATGTTGCTCA
AGTGACAGAAGACCATTGCTTGAAATGAAC TGCTTGAGAGATTTCCAG
AGAAGACGCTGAACATCATCCGCAAAAAATGTGTTGACGAAGGCGCTTGGAACAGA
20 CCAGTTAGTCAGTATTGACACCCGTTCTTGATATAGAACCCGGAGACAAACTGCTT
CTATGTTCTGACGGACTGACAATAAAGTGGAAAGGACTGAGTTAAAGACATCCTG
CAAAGCGATTAGCTCCTCAGGAAAAAGTAAACCTGCTTGAGACAAAGCCAATCAG
AATGGCGGAGAAGACAACATTACAGCAGTTGCTTGAGCTTGCTTACAAGTTGAAG
AGGGTGAAGATCAGTGC (SEQ ID NO:29).

25

The deduced amino acid sequence of the *prpC* protein is:

MLTALKTDTGKIRQHNEDDAGIFKGKDEFILAVVADGMGGHLAGDVASKMAVKAMGEKW
NEAETIPTAPSECEKWLIEQILSVNSKIYDHQAHEECQGMGTTIVCALFTGKTVSVAHIG
30 DSRCYLLQDDDFVQVTEDHSLVNELVRTGEISREDAEHPRKNVLTKALGTDQLVSIIDTR
SFDIEPGDKLLLCSDGLTNKVEGTELKDILQSDSAPQEKVNLVDKANQNGGEDNITAVLL
ELALQVEEGEDQC (SEQ ID NO:30).

35

Additionally, the coding region is found at about 1649684-1650445 bp of the *B.*

subtilis 168 chromosome.

The *rocA* coding sequence of the *rocA* protein (pyrroline-5 carboxylate dehydrogenase) of *B. subtilis* 168 is shown below:

40

ATGACAGTCACATACGCGCACGAACCATTACCGATTTACGGAAGCAAAGAATAAAA
CTGCATTGGGAGTCATTGGCCTTGAAACACTCAGCTGGCAAGCATTATCCGC
TTGTCATAAATGGAGAAAAAATTGAAACGGACCGAAAATCATTCTATTAAACCCGGC
AAATAAAGAAGAGATCATTGGGTACCGTCTACAGCGGATCAAGAGCTGCTGAAAA
AGCGATGCAAGCCGCATTGCAGGCATTGATTCTGGAAAAAAACAAAGACCGGAGCA
CCGCGAAATATTCTTTAAGGCAGCGGTATTTGCGCAGAAGAAAGCATGAATT
45 TCAAGCTATCTTGTGAAGGAAGCAGGAAAACCGTGGAAGGAAGCAGATGCGGACAC
GGCTGAAGCGATAGACTTTAGAGTTCTACGCGCGCCAAATGTTAAAGCTCAAGGA
AGGGGCTCCGGTGAAGAGCCGTGCTGGCGAGGTCAATCAATATCATTACGAAGCGC
TTGGCGTCGGCATCGTCAATTCTCCATTAACTTCCCGCTCGCGATTATGGCGGGAA
CAGCGGTGGCAGCGATTGTGACAGGAAATACGATTCTCTTAAACCGGCTGACGCAG

- 50 -

5 CCCCGGTAGTGGCAGCAAATTGTCGAGGTATGGAGGAAGCGGGCTGCCAAC
 GGCCTCTGAATTACATTCCGGAGATGGTCCGGAGATCGGTGATTCTTAGTTGAG
 CATCCGAAGACACGGTTGTCTCATTACAGGTCGGCTGCACTGGCTGCCGGATT
 TATGAGCGAGCTGCCAAGTGCAGCCGGCCAAAATGGCTAAACGGTAATTGC
 10 AGAAATGGCGGAAAAGACACAGTGCTGTGACAAGGACGCTGATCTGACCTTG
 TGCACTCTATCGTATTAGCATTGGATATTAGGACAGAAGTGTCTGCGGGC
 TCCCAGCGGGTCAATTGATCAGGATGTGATGAAAGTGGTGGAAAAAGCTGTGGCG
 CTGACCAAAACGCTGACTGTCGGCAATCCAGAAGATCCTGATACGTATATGGTCCC
 GTGATTGATGAAAGCATCCTACAACAAAGTGTGAAATACATTGAAATCGGCAAATCTG
 15 AAGGCAAGCTATTGGCCGGAGAAGGCATGATTCAAAAGGCTACTTATTAGC
 CGACGATCTTGAGATGTTGATGAAAACGCCGCTTGATGCAGGAAGAAATTTCG
 GCCCGGTTGTTGCGATTGCAAAGCGCGTGATTCGATCATATGCTGGAGATTGCCA
 ATAACACGGAATAACGGATTAACAGGTGCGCTCTGACGAAAAACCGTGCACATTG
 AACGGCGCGAGGATTCCATGTCGGAAACCTATTTAACAGAGGATGTACCG
 20 GAGCAATTGTCGGCTATCAGCGTTGGCGTTTAATATGTCAGGAACAGACTCAA
 AAGCAGGCGGTCCCGATTACTTAATTCTCATATGCAAGCCAAAACAACGTCCGAAG
 CTTT (SEQ ID NO:31).

The deduced amino acid sequence of the RocA protein is:

20 MTVTYAHEPFTDFTEAKNKTAFGESLAFVNTQLGHYPLVINGEKIETDRKIISNPANK
 EIIIGYASTADQELAEKAMQAALQAFDSWKKQRPEHRANILFKAAAILRRRKHEFSSYLV
 KEAGKPWKEADADTAEAIDLEFYARQMLKLKEGAPVKSRAGEVNQYHAEALGVGIVISP
 FNFPLAIMAGTAVAAIVGNTILLKPADAAPVVAKFVEVMEAGLPNGVLNYIPGDGAEIG
 25 DFLVEHPKTRFVSGTGSRAVGCRYERAALKVQPGQKWLKRVIAEMGGKDTVLVDKDADL
 DLAASSIVYSAFGYSGQKCSAGSRAVIHQDVYDEVVEKAVALTKTLTVGNPEDPTYMG
 PVIHEASYNKVMKYIEIGKSEGKLLAGGEKGDDSKGYFIQPTIFADVDENARLMQEEIFGPV
 VAICKARDFDHMLEIANNEYGLTGALLTKNRAHIERAREDFHVGNLYFNRGCTGAIVGY
 QPFGGFNMSGTDKAGGPDYLILHMQAKTTSEAF (SEQ ID NO:32).

30 Additionally, the coding region is found at about 3877991-3879535 bp of the *B.*
subtilis 168 chromosome.

35 The *rocD* coding sequence of the *rocD* protein (ornithine aminotransferase) of *B.*
subtilis 168 is shown below:

40 ATGACAGCTTATCTAAATCCAAAGAAATTATTGATCAGACGTCTCATTACGGAGCCA
 ACAATTATCACCCGCTCCGATTGTTATTCTGAAGCGCTGGGTGCTTGGTAAAGG
 ACCCGGAAGGCAATGAATATATGGATATGCTGAGTGCCTACTCTCGGGTAAACCAAGG
 GGCACAGACACCCGAAAATCATTAGGCATTAAAGGATCAGGCTGATAAAATCACCC
 TCACGTACGCGCGTTTCATAACGATCAGCTGGCCGTTTACGAAAAAACAGCTAA
 ACTGACAGGAAAGAGATGATTCTGCCATGAATACAGGAGCCGAAGCGGTTGAATC
 CGCGGTGAAAGCGCGAGACGCTGGCGTATGAAGTGAAGGGCGTAGCTGACAAT
 CAAGCGGAAATTATCGCATGTGTCGGAACTTCCACGGCCGACGATGCTGGCGGT
 45 ATCTCTTCTCTGAAGAGGAATATAACGAGGATTGGCCGATGCTTCCAGGAATC
 AAACCTATTCTTACGGCGATGTGGAAGCGCTTCGACAGGCCATTACGCCAATACA
 CGGGCATTCTGTTGAACCGATTCAAGGCGAAGCGGGCATTGTGATTCCGCTGAA
 GGATTTTACAGGAAGCGGGCGATTGTAAGGAAGAGAATGTCTGTTATTGCG
 GATGAAATTCAACGGGTCTGGACGTACAGGCAAGACGTTGCTGTGACTGGGA
 50 CGGCATTGTTCCGGATATGTATATCTGGGCAAAGCGCTGGCGGGTGTGTTCCC
 GATCTCTGCATTGGCGGACCGCGAGATCCTAGGCGTGTAAACCCTGGCTCACA

- 51 -

CGGCTAACATTGGTGGAAACCCGCTTGCATGTGCAGTGTCTATCGCTTCATTAGAA
 GTGCTGGAGGATGAAAAGCTGGCGGATCGTTCTCTGAACCTGGTAAATACTTAAA
 AGCGAGCTTGAGAGTATTGACAGCCCCTGTCACTAAAGAAGTCCGCCAGAGGGCT
 5 GTTTATCGGTGTTGAATTGACTGAAGCAGCACGTCCGTATTGTGAGCGTTGAAGGA
 AGAGGGACTTTATGCAAGGAAACGCATGATACAGTCATTGTTGCACCGCCATTA
 ATCATTTCAAAGAGGACTGGATTGGCGATAGAGAAAATTAAGCACGTGCTGCGA
 AACGCA (SEQ ID NO:33).

The deduced amino acid sequence of the RocD protein is:

10 MTALSKSKEIIDQTSHYGANHYPLPIVISEALGAWVKDPEGNEYMDMLSAYSAVNQGHR
 HPKIIQALKDQADKITLTSRAFHNDQLGPFYEKTAKLTKEMILPMNTGAEAVESAVKAAR
 RWAYEVKGVDNQAEIIACVGNFHGRMLAVSLSSEEEYKRGFGPMLPGIKLIPYGDVEA
 LRQAITPNTAAFLFEPIQGEAGIVPPPEGFLQEAAAICKEENVLFIADEIQTGLGRTGK
 15 TFACDWDGIVPDMDYILGKALGGGVFPISCIADREILGVFNPGSHGSTFGGNPLACAVSI
 ASLEVLEDEKLADRSLELGEYFKSELESIDSPVIKEVRGRGLFIGVELTEARPYCERLK
 EEGLLCKETHDTVIRFAPPLIISKEDLDWAIEKIKHVLRNA (SEQ ID NO:34).

20 Additionally, the coding region is found at about 4143328-4144530 bp of the *B. subtilis* 168 chromosome.

The *rocF* coding sequence of the *rocF* protein (arginase) of *B. subtilis* 168 is shown below:

25 ATGGATAAAACGATTCGGTTATTGGAATGCCAATGGATTAGGACAAGCACGACGC
 GGAGTGGATATGGGCCCGAGTGCCATCCGGTACGCTCATCTGATCGAGAGGGCTGTC
 AGACATGGGTATACGGTTGAAGATCTCGGTGACATTCCGATCAATCGCGAAAAAAAT
 CAAAAATGACGAGGAACCTGAAAAACCTGAATTCCGTTTGGCGGGAAATGAAAACCT
 CGCGCAAAAGGTCAACAAAGTCATTGAAGAGAAAAAAATCCCGTTGTCCTGGCGG
 30 TGACCACAGTATTGCATCGGCACGCTTGCAGGCACAGCGAAGCATTACGATAATCT
 CGGCGTCATCTGGTATGACGCGCACGGCGATTGAATACACTTGAACACTTCACCATC
 GGGCAATATTCA CGGCATGCCGCTCGCGGTACGCCTAGGCATTGGCCACGAGTCAC
 TGGTTAACCTTGAGGCTACGCGCCTAAATCAAACCGAAAACGTCGTACATTG
 GCGCCCCGTACTTGATGAAGGGGAGCGCAAGTACATTAAGGAAAGCGGCATGAAG
 35 GTGTACACAATGCACGAAATCGATCGTCTGGCATGACAAAGGTATTGAAGAAAC
 CTTGATTATTTATCAGCATGTGATGGCGTCCATCTGAGCCTTGATCTGGACGGACTTG
 ATCCGAACGACGCACCGGGTCTCGGAACCCCTGTCGTGGCGGCATCAGCTACCGG
 GAGAGCCATTGGCTATGGAATGCTGTATGACGCAGGCATATTACCTCAGCCGAA
 TTCGTTGAGGTTAACCGATCCTGATCACAAAAACAAAACGGCAAAACAGCAGTA
 40 GAGCTCGTAGAATCCCTGTTAGGGAGAAGCTGCTG (SEQ ID NO:35).

The deduced amino acid sequence of the RocF protein:

45 MDKTISVGMPMDLGQARRGVDMGPSAIRYAHIERLSDMGTVEDLGDIPINREKIKND
 EELKNLNSVLAGNEKLAQKVNKVIEEKKFPLVLGGDHSAIGTLAGTAKHYDNLGVWIYD
 AHGDLNTLETSPSGNIHGMPLAVSLGIGHESLVNLEGYAPKIKPENVVIIGARSLEGER
 KYIKESGMKVYT MHEIDRLG MTKVIEETLDYLSACDGVHLSLDLDGLDPNDAPGVGTPVV
 GGISYRESHLAMEMLYDAGITS AEFVEVNPI LDHKNKTGKTA VELVESLLGKLL (SEQ ID
 50 NO:36).

- 52 -

Additionally, the coding region is found at about 4140738-4141625 bp of the *B. subtilis* 168 chromosome.

The *Tdh* coding sequence of the *Tdh* protein (threonine 3-dehydrogenase) of *B. subtilis* 168 is shown below:

ATGCAGAGTGGAAAGATGAAAGCTCTAATGAAAAAGGACGGGGCGTTGGTGCTGT
 GCTGACTGAAGTCCCATTCCCTGAGATTGATAAACATGAAGTCCTCATAAAAGTAAA
 GCCGCTCCATATCGGGCACGGATGCCACATTATAATTGGGATCAATGGGCACGT
 CAGAGAATCAAACACCCTATGTTTCCGGCCATGAGTTAGCAGCGGCATCGTAGAGGGC
 10 GTGGGAGAGAATGTCAGCAGTGTAAAAGTGGGAGAGTATGTGTCGCGGAAACACA
 CATTGTCGTGGTGAATGTGTCCTGCCTAACAGGAAAATCTATGTGTGTACCAAT
 ACTGCTATAATCGGAGTGGACACGGCAGGCTGTTTGCAGGAGTATGTAAGTTCCA
 GCTGATAAACATTGGAGAAATCCCGCTGATATGGACCCGTCATTGCTTCCATTCAAG
 AGCCTTTAGGAAATGCAGTTACCGTACTCGAGAGGCCAGCCTGCAGGAGGAACGA
 15 CTGCAGTCATTGGATCGGACCGATTGGCTTATGGCTGTTGGGTTGCAAAAGCAG
 CAGGAGCTCTCAGGTGATAGCGATTGATAAGAATGAATAACAGGCTGAGGCTTGCAA
 AACAAATGGGAGCGACTTGTACTGTTCTATTGAAAAAGAAGACCCGCTCAAAATTGT
 AAGCGCTTAACGAGTGGAGAAGGAGCAGATCTGTTGTGAGATGTCGGGCCATCC
 20 CTCAGCGATTGCCAACATCCGGTACAATTGATTGACGAATAAGTGGTATTAAAGG
 GCTTACCATCCAAGGAATCACAGGAAGAAAATGTTTCAACATGGCGCCAGGTGTC
 TCAGTTGATCAGTTAACATGATCGATCTGCACCTGTTATTACCCATCAGTTCCAT
 TAGAGGAGTTGAAAAGGTTCGAACTGATGAGAAGCGGGAGTGCAGGAAAGTAA
 TTTAATTCCA (SEQ ID NO:37).

25

The deduced amino acid sequence of the *Tdh* protein is:

MQSGKMKALMKKDGA~~F~~GA~~V~~L~~T~~E~~P~~I~~D~~KHEVLIKVKAAASICGTDVHIYNWDQWARQRI
 KTPYVFGHEFSGIVEGVGENVSSVKVGEYVSAETHIVCGECVPCLGKSHVCTNTAIIGV
 30 DTAGCFAEYVKVPADNIWRNPADMDPSIASIQEPLGN~~A~~H~~T~~VLESQPAGGTTAVIGCGPI
 GLMAVAVAKAAGASQVIAIDKNEYRLRLAKQM~~G~~ATCTVIEKEDPLKIVSALTSGEGADLV
 CEMSGHPSAIAQGLAMAANGGRFHILSLPEHPVTIDLTKVVF~~K~~GLTIQGITGRKMFSTW
 RQVSQLISSNMIDLAPVITHQFPLEEFKGELMRSGQCGKVILIP (SEQ ID NO:38).

35

Additionally, the coding region is found at about 1769731 – 1770771 bp of the *B. subtilis* 168 chromosome.

The coding sequences for the tryptophan operon regulatory region and genes *trpE* (SEQ ID NO:48), *trpD* (SEQ ID NO:46), *trpC* (SEQ ID NO:44), *trpF* (SEQ ID NO:50), *trpB* (SEQ ID NO:42), and *trpA* (SEQ ID NO:40) are shown below. The operon regulatory region is underlined. The *trpE* start (ATG) is shown in bold, followed as well by the *trpD*, *trpC* *trpF*, *trpB*, and *trpA* starts (also indicated in bold, in the order shown).

TAATACGATAAGAACAGCTTAGAAATACACAAAGAGTGTGTATAAGCAATTAGAATGA
GTTGAGTTAGAGAATAGGGTAGCAGAGAATGAGTTAGTTGAGCTGAGACATTATGTT
 45 TATTCTACCCAAAAGAAGTCTTCTTGGGTTATTTGTTATATAGTATTTATCCTCT
CATGCCATCTCTCATTCTCCATAGGAGTGAGAGCAATGAATTCCAATCAA

- 53 -

ACATTTCCGCATTTAGAGGACAGCTTGTCCCACCACACGATACCGATTGTGGAGAC
CTTCACAGTCGATACTGACACCCATTCAAATGATAGAGAAGCTGACAGGGAGATT
ACGTATCTTCTTGAAGCAAGGACGATACATCCACTGGTCCAGATATTGTTATCG
GCCTGAATCCATTCTCACAATTAAAGAACAGAGCAGGGCCGTTTCGGCCGCTGATC
5 AGGACAGCAAATCTTTACACAGGAATGAACCTAAAAGAACAGTGCTGAACGGATGAA
TACCACATACAAAATCAAAACACCTGAGCTGGCATTCTTGTGGCGAGCTGTC
GGGTACTTAAGCTATGATATGATCCCCTGATTGAGCCTCTGTTCTGCATACCA
AAGAAACAGACATGGAAAAGTGTATGCTGTTGCCCCACATTAATTGCGTATGA
TCATGAAACCAAAACGTCCACTTTATCCAATATGCAAGGCTACTGGAGAGGAAACA
10 AAAAACGAAAAATGGATGTATTCCATCAAATCATCTGGAGCTTCAAATCTCATTGA
AAAAATGATGGACCAAAAAACATAAAAGAGCTTTCTGCTGATTGATACAAAGA
CACCCAGCTTGAGACAGTATCTCTAATTATGAAAAATCGGCTTTATGGCTGATGTA
GAAAAAAATCAAAGCTATATAAAAGCAGGCGATATCTCCAGGGTGTGTTATCACAAA
AATTGAGGTGCCGATAAAAGCAGATGCTTGAGTTACCGAGTGCTTAGGATCGT
15 CAATCCTCGCCGTATATGATTATGAAACTGCTAGACAGAGAAATAGTCGGCAGC
TCTCCGGAACGGTTAACACGTTCAAGACGGGCACCTAGAAATCCATCCGATTGCC
GGTACGAGAAAACGCGGTGCAGACAAAGCTGAAGATGAGAGACTGAAGGTTGAGCT
CATGAAGGATGAAAAGAAAAGCAGGAGCATTACATGCTCGTTGATCTGCCGAAA
CGATATCGGCAGAGTAGCAGAGTATGGTTCTGTTGCCCCAGTTAACAGGC
20 TGTTCCCTTTCACATGTCATGCACATTATCTCGGTGGTTACAGGGCGATTGAAAAAA
GGGGTTCATCCTGTCGATGCACTGATGTCGCTTCCCGGGGGGACTTAAACAGGC
GCACCCAAAATCCGTGCCATGCAAGCTTGCAAGAACTCGAGCCAACACCGAGAGAG
ACATACGGAGGGTGTATTGCTACATTGGTTGACGGAAATATCGACTCTGTATTA
CGATTTCGCACGATGAGTGTAAAGAACGGTGTGCATCGATAACAGGCAGGTGCTGGC
25 ATTGTTGCTGATTCTGTCGGGAAGCCGAATACGAAGAAAGCTGTAATAAGCCGGT
GCGCTGCTGAAAACGATTCAATTGCAAGAACATGTTCATAGCAAGGAGGATAAA
GCTGATGAAACAGATTCTACAATTGTCGTTGACGGAAAACCTTACTGCCGGTGA
GGCTGAAACGCTGATGAATATGATGATGGCAGCGGAAATGACTCCTCTGAAATGGG
GGGGATATTGTCATTCTGCTCATCGGGGGAGACGCCAGAACAGAGCTTGCAGGGTT
30 TTGTGAAGGCAATGCGGGCACACGCTTACAGTCGATGGACTTCTGATATTGTTG
ATACATGCGGAACAGGGGGAGACGGTATTCCACTTTAATATCTAACGGCCTCGG
CAATTGTTGCCCTCGGCAGCTGGTGCAGAAATCGCTAACGATGGCAATCGCTCTGTCT
CTTCTAAAAGCGGAAGCGCTGATGTTAGAGGAGCTAGAGGTTCTATTCAAACCCAC
TCCCAGAAAGGTCAAAGCAGCATTGAAACAAACATGGGATTCTTTGCCCG
35 CTTTACCATCGCTATGAAACATGTCAGGACTAGAAAAGAGCTAGGTTTCAAGAA
CGGTATTAAATCTGCTTGGCCGCTCAGCAATCCTTACAGGCCAGCGTCAGGTGA
TTGGGGTCTATTCTGTTGAAAAGCTGGACTGATGGCAAGCGCACTGGAGACGTTTC
AGCCGAAGCACGTTATGTTGATCAAGCCGTACGGTTAGATGAGCTTCAATTAC
AGCACCGACCGACGTGATTGAATTAAAGGACGGAGAGCGCCGGAGTACCGTT
40 CACCCGAAGATTGCGTTCAAAATGGCAGACTTGAAGATTACAGGTGCAGTCCTCC
GAAAGAGAGCGCTTATCTCATTCAAGAATATTGAAAATAAAAGCAGCAGTCCGCT
TTATCTATTACGGCTTTAATGCGGGTGCTGCGATTACAGGCCGGATTACCGCCT
CACTGAAGGAAGGAACGGAGCTGGCTTAGAGACGATTACAAGCGGAGGGCCTGCC
GCGCAGCTGAAACGACTAAAGCAGAAAGAGGAAGAGATCTATGCTGAAAAATCAT
45 CAAACAAAAGAAAGAAGAAGTGAAAACACTGGTTCTGCCGGTAGAGCAGCCTTCGA
GAAACGTTCAATTAAAGGAGGCGCCGGCAAGCCGAATCGGTTATCGGGTTGATTGC
CGAAGTGAAGAAAGCATGCCGTAAAAGGGCTTATTAAGAGGATTGTTACCTGT
GCAGATTGCAAAGACTATGAGGCTGCGAAGGCAGATGCGATTCCGTTAACAGA
CACCCGTTTCAAGGGAAAACAGCTATTATCAGACGTAAGCGTGCTGTTCG
50 ATTCCTGTACTTAGAAAAGATTATTGATTCTCTCAAGTAGAGGAATCAAGAAG
AATCGGAGCGGATGCCATTGTTAATCGGCAGGGTGTGATCCCTACACCTTCA
GAATTATATCTGAAGCAGGTGAAAAGGGATGGACGTGTTAGTGGAGGTTCATGAT
GCATCAACGCTAGAACAAATATTGAAAGTGTACACCCGACATTCTGGCGTAAATA

- 54 -

ATCGAAACCTAAAACGTTGAAACATCTGTAAGCAGACAGAACAAATCGCATCTCT
 CGTTCGAAAGAACATCCTGCTTGTCAGCGAAAGCGGAATCGGTTCTTAGAACATTAA
 ACATTGTCAATGAACATGGGGCGCGAGCTGTACTTATCGGTGAATCATTGATGAGA
 CAAACTCTCAGCGTAAAGCAATCCATGCTTGTAGGGAGTGAGGTTGTGAAGAAA
 5 CCGGCATTAAAATATTGCCGTATCGGTACTAAAGGATTGAGCTTGCGCGCGAA
 TCACAGGCTGATTACCTAGGATTATTTGCTGAAAGCAAACGAAAAGTATCTCCGG
 AAGATGTGAAAAAAATGGCTGAACCAAGTCTGTGCAAAAACAGGTTGCAGGTGTTT
 TGTTAATGAATCAATAGAGACGATGTCACGTATTGCCAAGAGCTTGAGCTCGACGTC
 ATTCACTACGGGTGATGAAAAACCGCGGATGTCGCTGCTTCGCAAGCTGACA
 10 GGCTGTGAAATATGGAAGGCCTCACCATCAAGATAACACAACCTCAAGAAATAGCC
 CGCTTAAAGATAATGTTGACGGCTTGTGATTGATTCATCTGAAAAGGGTCTAGAG
 GCGGAACCTGGTGTGCATTTCTGGACTGTGTGCCGAATATCAGCAGGCGGCTA
 TTGGTAAACGCTGCTTATCGCTGGCGCGTGAATCCGGATAGCATCACACGCCAT
 TGAAATGGCAGCCAGAAGGAATTGACCTTGGCAGCGGAATTGAAAAAAACGGACAAA
 15 AAGATCAGAATCTGATGAGGCTTTAGAAGAAAGGATGAACCGATATGTATCCATATC
 CGAATGAAATAGGCAGATACGGTGATTTGGCGGAAAGTTGTTCCGAAACACTCA
 TGCAGCCGTTAGATGAAATACAAACAGCATTAAACAAATCAAGGATGATCCCCTT
 TCGTGAAGAGTATTATAAGCTGTTAAAGGACTATTCCGGACGCCGACTGCATTAACA
 TACGCTGATCGAGTCACTGAATACTTAGGCCGCGAAAATCTATTGAAACGAGAA
 20 GATTAAACCATAACAGGTTCTCATAAAATCAATAATGCGCTAGGTCAAGCGCTGCTT
 CTAAAAAAATGGCAAAACGAAAATCATTGCTGAAACCGGTGCCGCCAGCATGGT
 TTGCCGCTGCAACAGTTGAGCCAAATTGGCTTCTGTACTGTGTTATGGGTGA
 AGAGGATGTTGCCGCCAGTCTCTGAACGTTCCGCATGAAGCTTCTGGAGCGGA
 GGTAGTGCCTGTAACAAGCGAACCGAACATTGAAGGATGCCACAAATGAGGCGA
 25 TCCGGTACTGGGT
 TCAGCATTGTGAGGATCACTTTATATGATTGGATCAGTTGTCGGCCCCATCCTTAT
 CCGCAAGTGGTCCGTGAATTCAAAAATGATCGGAGAGGAAGCGAAGGATCAGTTG
 AAACGTATTGAAGGCACATGCCTGATAAAGTAGTGGCATGTGTAGGCGGAGGAAGC
 AATGCGATGGGTATGTTCAAGGCTTTAAATGAAGATGTTGAACTGATCGCGCTG
 30 AAGCAGCAGGAAAAGGAATTGATAACACCTCTCATGCCGCCACTATTCGAAAGGAA
 CCGTAGGGGTTATTACGGTTATTGACTTATCTCATTCAAGGATGAGTCGGGCCAAAT
 TATTGAGCCCTACTCTATTCAAGCCGCTCGACTATCCTGGAATCGTCCGGAGCA
 TGCAATTGCAAAAGCGGCCGTCACTTATGACAGTATAACCGATGAAGAAC
 GGTGGATGCATTAAAGCTTGTCAAGAAAAGAGGGATTGGCCGCAATGAATC
 35 TGCCCATGCGTTAGCAGAACGATTCAAACCTGCCAAGGAATTGGATCGCGCTCAACT
 CATTCTCGTCTGTTATCAGGCCGGGGAGACAAGGATGTCACACATTAATGAATGTA
 TTGGAAGAAGAGGTGAAAGCCCAGTTAAATTGGATCTCAACCATCAGAAAATTG
 TTTATCCGTTATTACGGCGGGCGATCCAGTTCTGAGGTTGATTGAACTGGCG
 AAGTCACTCCAAAAGCAGGCGCCACAGCATTGGAGCTTGGTGTGATCAACTCTGAC
 40 CCGCTTCAGACGGTCCGGTGTCCAGCGGGCTTCAAAGCGGGCGCTTGTGATCAAGG
 AATGAATATCGTAAAGGCAATCGAATTAGGCGGAGAAATGAAAAAAACGGAGTGAA
 TATTCCGATTATCCTCTTACGTATTATAATCCTGTTACAATTGAACAAAGAACAA
 TTTCGCTTACTGCGGGAAAATCATATTGACGGTCTGCTTCCGGATCTGCCATTA
 GAAGAAAGCAACAGCCTCAAGAGGAATGTAAGGCCATGAGGTGACGTATATTCTT
 45 TAGTTGCGCCGACAAGCGAAAGCCGTTGAAAACATTATTGAACAAAGCCGAGGGGT
 TCGTCTACTGTGATCTTCTCTGGGTGTGACCGGTGTCGCAATGAGTTCAATTCTC
 CGTGTACCCGTTATCGTACTGTGAAAGAACATCTCAGCACTGTTCCGGTTGCTGAGG
 GTTCGGTATATCAAACCGTGAACAGGTCAAAAGATGAATGAAATTAGTGACGGTGTC
 GTAGTGGGAAGTGCCTCGTCAGAAAATAGAAGAATTAAAGGACCGGCTCATCAGC
 50 GCTGAAACGAGAAATCAGGCGCTGCAGGAGTTGAGGATTATGCAATGGCGTTAGC
 GGCTTGACAGTTAAA (SEQ ID NO:39).

- 55 -

The deduced TrpA protein (tryptophan synthase (alpha subunit)) sequence is:

5 MFKLDLQPSEKLFIPFITAGDPVPEVSIELAKSLQKAGATALELGVAYSDPLADGPVIQR
 ASKRALDQGMNIVKAIELGGEMKKNGVNIPIIIFTYYNPVLQLNKEYFFALLRENHIDGL
 LVPDLPLEESNSLQEECKSHEVTYISLVAFTSESRLKTIIEQQAEGFVYCVSSLGVTGVRN
 EFNSSVYPFIRTVKNLSTPVAVGFGISNREQVKMNEISDGVVVGASALVRKIEELKDRL
 ISAETRNQALQEFDYAMAFSGLYSLK (SEQ ID NO:41).

10 The deduced TrpB protein (tryptophan synthase (beta subunit)) sequence is:

15 MYPYPNEIGRYGDFGGKFV рет LMQPLDEIQTAFKQIKDDPAFREEYYKLLKDYSRPTA
 LTYADRVT ЕYLGGAКIYLKREDLNHTGSHKINNAЛGQALLAKKMGTКIIAETGAGQHGVA
 AATVAAKFGFСTVFMGEEDVARQSLNVFRMKLLGAEVVPVTSGNGTLKDATNEAIRYW
 VQHCEDHFYMIGSVVGPHYPQVREFQKMIGEEAKDQLKRIEGTMPDKVVAСVGGGS
 NAMGMFQAFLNEDVELIGAEAAGKGIDTPLHAATISKGTGVVIHGSLTЫLIQDEFGQIEPY
 SISAGLDYPGIGPEHAYLHKSGRVTYDSITDEEAVDALKLSEKEGILPAIESAHALAKAKL
 AKGMDRGQLILVLSGRGDKDVNTLMNVLEEVKAHV (SEQ ID NO:43).

20 The deduced TrpC protein indol-3-glycerol phosphate synthase) sequence is:

25 MLEKIIKQKKEEVKTLVLPVEQPFEKRSFKEAPASNRFIGLIAEVKKASPSKGLIKEDF
 VPVQIAKDYEAAKADAISVLTDTFFQGENSYLSDVKRAVSIPVLRKDFIIDSLQVEESR
 RIGADAIIЛGEVLDPLHLHELYLEAGEKGMDVLVEHDASTLEQILKVFTPDIЛGVNRR
 NLKTFETSVKQTEQIASLPKESLLVSESGIGSLEHLTFVNEHГARAVLIGESLMRQTSQ
 RKAIHALFRE (SEQ ID NO:45).

30 The deduced TrpD protein (anthranilate phosphoribosyltransferase) sequence is:

35 MNRFLQLCVDGKLTAGEAETLMNMMMAEМTPSEMGGILSILAHRGETPEELAGFVKA
 MRAHALTVДGLPDIVDTCTGGDGISTFNISTAIVASAAGAKIAKHGNRSVSSKSGSAD
 VLEELEVSIQTTPEKVKSSIETNNMGLFAPLYHSSMKHVAGTRKELGFRTVFNLLGPLSN
 PLQAKRQVIGVYSVEKAGLMSALETFQPKHVMFVSSRDGLDELSITAPTDVIELKDGER
 REYTVSPEDFGFTNGRLEDLQVQSPKESAYLIQNIFENKSSSALSITAFNAGAAIYTAGIT
 ASLKEГTELATEITITSGGAAAQLERLKQKEЕEIYA (SEQ ID NO:47).

40 The deduced TrpE protein (anthranilate synthase) sequence is:

45 MNFQSNIASFEDSLSHHTIPIVETFTVDTLTPIQMIEKLDREITYLLESKDDTSTSRY
 SFIGLNPFЛTIKEEQGRFSAADQDSKSLYTGNELKEVLNWМNTTYKIKTPELGIPFVGGA
 VGYLSYDMIPLIEPSVPSHTKETDMЕKCMFVCRTLIAYDHETKNVHFQYARLTGEETK
 NEKMDVHFHQHLELQNLIЕKMDQKNIKEFLSADSYKTPSFETVSSNYEKSАFMADVEK
 IKSYIKAGDIFQGVLSQKFEVPIKADAFELYRVLRIVNPSPYМYYMКLLDREIVGSSPERLIH
 VQDGHLEIHPИAGTRKRGADKAEDERLKVELMKDEKEKAЕHYMLVDLARNDIGRVAЕYГ
 SVSVPEFTKIVSFHVMHIISV/TGRLKKGVHPVDALMSAFPAGTLTGAPKIRAMQLLQEL
 EPTPRETYGGCIAYIGFDGNIIDSCITIRMSVKNGVASIQAGAGIVADSVPEAEYEESCНKA
 GALLKTIHIAEDMFHSKEDKADEQISTIVR (SEQ ID NO:49).

50 The deduced TrpF protein (phosphoribosyl anthranilate isomerase) sequence is:

- 56 -

MKKPALKYCGIRSLKDLQLAAESQADYLGFIFAESKRKVSPEDVKKWLNRVKEKQVAG
VFVNESIETMSRIAKSLKLDVIQLHGDEKPADVAALRKLTGCEIWALKHHQDNTTQEIA
RFDNVDFVIDSSVKGSRGGTGVAFSWDCVPEYQQAAIGKRCFIAGGVNPDSITRLLK
PEGIDLASGIEKNGQKDQNLMRLLERMNRYVSISE (SEQ ID NO:51).

5

Additionally, the coding region is found at about 2370707 bp to 2376834 bp
(first bp = 2376834; last bp = 2370707) bp of the *B. subtilis* 168 chromosome.

10 The *ycgM* coding sequence of the *ycgM* protein (similar to proline oxidase) of *B. subtilis* 168 is shown below:

15 GTGATCACAAAGAGATTTTCTTATTTTATCCAAAAGCGGCTTCTCAATAAAATGGC
GAGGAACCTGGGAAGTCGGTAGCAGCGGGTAAAATTATCGGCAGGAATGACTTA
ACAGTTCAATCCCGACCATTGACAGCTAACAGCCAAGGCTTGTCACTGTCGA
TCATTTAGGCAGTTGTAAACAGCGCCGAGGTCGCACGGGAGCGTACGGAAGAGT
GCATTCAAACCATTGCGACCATCGCGGATCAGGAGCTGAACACTCACACGTTCTTAAA
AATGACGTCTTCTGGATATAGATATGGATTGGTGTATGAAAATATGACAAAAAA
20 TCCTTCAGACGGCGAGAACATAAAATCATGGTCACCATTGACATGGAGGACGAAG
TCAGATGCCAGAAAACGCTTGATATTTCAAAGATTCAAGAAATACGAGCATGT
GAGCACAGTGTGCAAGCCTATCTGTACCGGACGGAAAAGACATTGACGATTGGA
25 TTCTTAAACCGTTCTCGCCTTGAAAAGGAGCTATAAAGAATCAGAAAAAGTA
GCTTCCCAGAAAAGCGATGTCGATGAAAATTACAAAAAAATCATCCGAAAGCAG
CTCTTAAACGGTCACTATACAGCGATTGCCACACATGACGACAAATGATCGACTTA
CAAAGCAGCTGCCAAGAACATGGCATTGCCAATGACAAGTTGAATTCAAGATGCT
GTACGGCATGCGTCGCAAACCCAGCTCAGCCTCGAAAAGAAGGTTATAACATGAG
30 AGTCTACCTGCCATACGGCGAGGATTGGTACGGCTACTTTATGAGACGCCCTG
ACGTCGGTCAAACATTGCATTGCTTCAAAGGAATGACAAAGAAG (SEQ ID NO:52).

The deduced amino acid sequence of the *YcgM* protein is:

35 MITRDFFLFLSKSGFLNKMARNWGSRVAAGKIIIGGNDNFNSSIPTIRQLNSQGLSVVDHL
GEFVNSAEVARERTEECIQTIATIADQELNSHVSLSKMTSLGLDIDMDLVENMTKILQTA
EKKHIMVTIDMEDEVRCQKTLDIFKDFRKKYEHVSTVLQAYLYRTEKDIDDDLSLPFLR
LVKGAYKESEKVAFPEKSDVDENYKKIIRKQLLNGHTAIATHDDKMIDFTKQLAKEHGI
ANDKFEFQMLYGMRSQTQLSLVKEGYNMRVYLPYGEDWYGYFMRRRAERPSNIAAFK
GMTKK (SEQ ID NO:53).

Additionally, the coding region is found at about 344111-345019 bp of the *B. subtilis* 168 chromosome.

40 The *ycgN* coding sequence of the *ycgN* protein (similar to 1-pyrroline-5-carboxylate dehydrogenase) of *B. subtilis* 168 is shown below:

45 ATGACAACACCTACAAACACGAGCCATTCAAAATTCCAAGATCAAAACTACGTGG
AAGCGTTAAAAAAGCGCTTGCAGACAGTAAGCGAATATTAGGAAAAGACTATCCGCT
TGTCATTAACGGCGAGAGAGTGGAAACGGAAGCGAAAATCGTTCAATCAACCCAGC
TGATAAAAGAAGAAGTCGTCGGCGAGTGTCAAAAGCGTCTCAAGAGCACGCTGAGC
AAGCGATTCAAGCGGCTGCAAAAGCATTGAAGAGTGGAGATACACGTCTCCTGAAG
AGAGAGCGGCTGTCCTGTCGCGCTGCTGCCAAAGTCCGAGAAGAAAATGAA

- 57 -

TTCTCAGCTTGCTTGTGAAAGAAGCAGGAAAGCCTTGGAACCGAGGCCGATGCCGAT
 ACGGCTGAAGCGATTGACTTCATGGAGTATTATGCACGCCAAATGATCGAACTGGCA
 AAAGGCAAACCGGTCAACAGCCGTGAAGGGCAGAAAAACCAATATGTATAACGCCG
 ACTGGAGTGACAGTCGTTATCCCGCCTTGAACCTTCTGTTGCGATCATGGCAGGC
 5 ACAACAGTGGCGCCGATCGTTACTGGAAACACAGTGGTTCTGAAACCTGCGAGTGCT
 ACACCTGTTATTGAGCAGAAAATTGTTGAGGTGCTTGAAGAGTCCGGATTGCCAAAAG
 GCGTAGTCAACTTGTCCGGAAAGCGGATCGGAAGTAGGCAGACTATCTGTTGACC
 ATCCGAAAACAAGCCTTATCACATTACGGGATCAAGAGAAGTTGGTACGAGAATT
 CGAACGCGCGGCGAAGGTTAGCCGGGCCAGCAGCATTAAAGCGTGTATCGCTG
 10 AAATGGGCGGTAAAGATACTGGTTGTTGATGAGGATGCGGACATTGAATTAGCGG
 CTCATCGATCTTACTTCAGCATTGGCTTGCAGGACAAAATGCTCTGCAGGTT
 ACGTGCAGTAGTTCATGAAAAGTGTATGATCAAGTATTAGAGCGTGTATTGAAATT
 ACGGAATCAAAGTAACAGCTAACCTGACAGTGCAGATGTTATATGGGACCTGTCA
 TTGACCAAGGTTCTTATGATAAAATTATGAGCTATATTGAGATCGGAAAACAGGAAGG
 15 GCGTTTAGTAAGCGGCGGTACTGGTGATGATTGAAAGGAACTTCATCAAACCGAC
 GATCTTCGCTGACCTTGATCCGAAAGCAAGACTCATGCAGGAAGAAATTTCGGACC
 TGTCGTTGCATTTGTAAGTGTCAAGACTTGTATGAAAGCTTTAGAAGTGGCAAACAAT
 ACTGAATATGGTTGACAGGCGCGGTTATCACAAACAACCGCAAGCACATCGAGCGT
 GCGAAACAGGAATTCCATGTCGAAACCTATACTTCAACCGCAACTGTACAGGTGCT
 20 ATCGTCGGCTACCATCCGTTGGCGCTTCAAAATGTCGGGAAACGGATTCAAAGCA
 GGCGGGCCGGATTACTGGCTCTGCATATGCAAGAAAAACATCAGTGAATGTT
 (SEQ ID NO:54).

The deduced amino acid sequence of YcgN protein is:

25 MTTPYKHEPFTNFQDQNYVEAFKKALATVSEYLKDYPLVINGERVETEAKIVSINPADK
 EEVVGRVSKASQEHAEQAIQAAAFAFEWRYTSPPEERAAVLFRAAKVRRRKHEFSALL
 VKEAGKPWNEADADTAEAIDFMEYYARQMIELAKGKPVNSREGEKNQYVYPTGTVVVI
 PPWNFLFAIMAGTTVAPIVTGNTVVLKPASATPVIAAKFVEVLEESGLPKGVVNFPVGSGS
 30 EVGDYLVDHPKTSLITFTGSREVGTRIFERAALKVQPGQQHLKRVIAEMGGKDTVVDEDA
 DIELAAQSIFTSAFGFAGQKCSAGSRAVHEKVDQVLERVIEITESKVTAKPDSADVYMG
 PVIDQGSYDKIMSYIEIGKQEGRLVSGGTGDDSKGYFIKPTIFADLDPKARLMQEEIFGPVV
 AFCKVSDFDEALEEVANNTEYGLTGAIVTNNRKHIERAKQEFHVGNLVFNRNCTGAIVGYH
 PFGGFKMSGTDSKAGGPDYLAHMQAKTISEMF (SEQ ID NO:55).

35 Additionally, the coding region is found at about 345039-346583 bp of the *B.*
subtilis 168 chromosome.

The *sigD* coding sequence of the *sigD* protein (RNA polymerase flagella, motility, chemotaxis and autolysis sigma factor) of *B. subtilis* 168 is shown below:

40 ATGCAATCCTTGAATTATGAAGATCAGGTGCTTGGACGCCCTGGAAAGAGTGGAAA
 GATCCTAAAGCCGGTGACGACTTAATGCGCCGTTACATGCCGCTTGTACATATCAT
 GTAGGCAGAATTCTGTCGGACTGCCAAATCAGTCATAAAGACGATCTTATGAGC
 CTTGGTATGCTGGTTATATGATGCCCTGAAAAATTGACCCCAGCCGGGACTAA
 45 AATTGATACCTACGCCCTCGTTAGAATTGCCGGCGCAATCATAGACGGGCTTCGTA
 AGAAGATTGGCTGCCAGAACCTCGCGGAAAAACAAAAAAGGTTGAAGCAGCAAT
 TGAAAAGCTTGAACAGCGGTATCTCGGAATGTATGCCCGCGGAAATTGCAGAGGA
 ACTCGGAATGACGGTACAGGATGTCGTCAACATGAATGAAGGTTTTTGCAAAT
 CTGCTGTCAATTGATGAAAAGCTCCATGATCAAGATGACGGGGAAAACATTCAAGTCA
 50 TGATCAGAGATGACAAAAATGTTCCGCGCTGAAGAAAAGATTATGAAGGATGAACTGAT
 TGCACAGCTGCGGAAAAATTCACGAACTCTGAAAAAGAACAGCTGGTTGTCAG

- 58 -

TTTGTCTACAAAGAGGAGTTGACACTGACAGAAATCGGACAAGTATTAAATCTTCT
ACGTCCCGCATATCTCAGATCCATTCAAAGCATTATTAAATTAAGAATCTGCTGG
AAAAAGTGATACAA (SEQ ID NO:56).

5 The deduced amino acid sequence of the SigD is:

MQSLNYEDQVLWTRKEWKDPKAGDDLMRRYMPPLVTYHGRISVGLPKSVHKDDLMS
LGMLGLYDALEKFDPSPRDLKFDTYASFRIRGAIIDGLRKEDWLPRTSREKTKVAAIEKL
EQRYLRNVSPAIAEELGMTVQDVVSTMNEGFFANLLSIDEKLHDQDDGENIQVMIRDDK
10 NVPPEEKIMKDELIAQLAEKIHELSEKEQLVSLFYKEELLTEIGQVNLSTSRSQIHSKA
LFKLKNLLEKVIQ (SEQ ID NO:57).

Additionally, the coding region is found at about 1715786-1716547 bp of the *B. subtilis* 168 chromosome.

15 As indicated above, it is contemplated that inactivated analogous genes found in other *Bacillus* hosts will find use in the present invention.

In some preferred embodiments, the host cell is a member of the genus *Bacillus*, while in some embodiments, the *Bacillus* strain of interest is alkalophilic. Numerous alkalophilic *Bacillus* strains are known (See e.g., U.S. Pat. 5,217,878; and Aunstrup et al.,
20 Proc IV IFS: Ferment. Technol. Today, 299-305 [1972]). In some preferred embodiments, the *Bacillus* strain of interest is an industrial *Bacillus* strain. Examples of industrial *Bacillus* strains include, but are not limited to *B. licheniformis*, *B. lentus*, *B. subtilis*, and *B. amyloliquefaciens*. In additional embodiments, the *Bacillus* host strain is selected from the group consisting of *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. thuringiensis*, *B. clausii*, and *B. megaterium*, as well as other organisms within the genus *Bacillus*, as discussed above. In some particularly preferred embodiments, *B. subtilis* is used. For example, U.S. Patents 5,264,366 and 4,760,025 (RE 34,606) describe various *Bacillus* host strains that find use in the present invention, although other suitable strains are contemplated for use in the 25 present invention.

An industrial strain may be a non-recombinant strain of a *Bacillus* sp., a mutant of a naturally occurring strain or a recombinant strain. Preferably, the host strain is a recombinant host strain wherein a polynucleotide encoding a polypeptide of interest has been introduced into the host. A further preferred host strain is a *Bacillus subtilis* host 30 strain and particularly a recombinant *Bacillus subtilis* host strain. Numerous *B. subtilis* strains are known, including but not limited to 1A6 (ATCC 39085), 168 (1A01), SB19, W23, Ts85, B637, PB1753 through PB1758, PB3360, JH642, 1A243 (ATCC 39,087), ATCC 21332, ATCC 6051, MI113, DE100 (ATCC 39,094), GX4931, PBT 110, and PEP 35 211strain (See e.g., Hoch et al., Genetics, 73:215-228 [1973]; U.S. Patent No. 4,450,235;

- 59 -

U.S. Patent No. 4,302,544; and EP 0134048). The use of *B. subtilis* as an expression host is further described by Palva *et al.* and others (See, Palva *et al.*, Gene 19:81-87 [1982]; also see Fahnstock and Fischer, J. Bacteriol., 165:796-804 [1986]; and Wang *et al.*, Gene 69:39-47 [1988]). .

5 Industrial protease producing *Bacillus* strains provide particularly preferred expression hosts. In some preferred embodiments, use of these strains in the present invention provides further enhancements in efficiency and protease production. Two general types of proteases are typically secreted by *Bacillus sp.*, namely neutral (or "metalloproteases") and alkaline (or "serine") proteases. Serine proteases are enzymes
10 which catalyze the hydrolysis of peptide bonds in which there is an essential serine residue at the active site. Serine proteases have molecular weights in the 25,000 to 30,000 range (See, Priest, Bacteriol. Rev., 41:711-753 [1977]). Subtilisin is a preferred serine protease for use in the present invention. A wide variety of *Bacillus* subtilisins have been identified and sequenced, for example, subtilisin 168, subtilisin BPN', subtilisin
15 Carlsberg, subtilisin DY, subtilisin 147 and subtilisin 309 (See e.g., EP 414279 B; WO 89/06279; and Stahl *et al.*, J. Bacteriol., 159:811-818 [1984]). In some embodiments of the present invention, the *Bacillus* host strains produce mutant (e.g., variant) proteases. Numerous references provide examples of variant proteases and reference (See e.g., WO 99/20770; WO 99/20726; WO 99/20769; WO 89/06279; RE 34,606; U.S. Patent No.
20 4,914,031; U.S. Patent No. 4,980,288; U.S. Patent No. 5,208,158; U.S. Patent No. 5,310,675; U.S. Patent No. 5,336,611; U.S. Patent No. 5,399,283; U.S. Patent No. 5,441,882; U.S. Patent No. 5,482,849; U.S. Patent No. 5,631,217; U.S. Patent No. 5,665,587; U.S. Patent No. 5,700,676; U.S. Patent No. 5,741,694; U.S. Patent No. 5,858,757; U.S. Patent No. 5,880,080; U.S. Patent No. 6,197,567; and U.S. Patent No.
25 6,218,165).

In yet another embodiment, a preferred *Bacillus* host is a *Bacillus sp.* that includes a mutation or deletion in at least one of the following genes, *degU*, *degS*, *degR* and *degQ*. Preferably the mutation is in a *degU* gene, and more preferably the mutation is *degU(Hy)32*. (See, Msadek *et al.*, J. Bacteriol., 172:824-834 [1990]; and Olmos *et al.*, Mol. Gen. Genet., 253:562-567 [1997]). A most preferred host strain is a *Bacillus subtilis* carrying a *degU32(Hy)* mutation. In a further embodiment, the *Bacillus* host comprises a mutation or deletion in *scoC4*, (See, Caldwell *et al.*, J. Bacteriol., 183:7329-7340 [2001]); *spolIE* (See, Arigoni *et al.*, Mol. Microbiol., 31:1407-1415 [1999]); *oppA* or other genes of the *opp* operon (See, Perego *et al.*, Mol. Microbiol., 5:173-185 [1991]). Indeed, it is contemplated that any mutation in the *opp* operon that causes the same phenotype as a

- 60 -

- mutation in the *oppA* gene will find use in some embodiments of the altered *Bacillus* strain of the present invention. In some embodiments, these mutations occur alone, while in other embodiments, combinations of mutations are present. In some embodiments, an altered *Bacillus* of the invention is obtained from a *Bacillus* host strain that already 5 includes a mutation to one or more of the above-mentioned genes. In alternate embodiments, an altered *Bacillus* of the invention is further engineered to include mutation of one or more of the above-mentioned genes.

In yet another embodiment, the incoming sequence comprises a selective marker located between two *loxP* sites (See, Kuhn and Torres, *Meth. Mol. Biol.*, 180:175-204 10 [2002]), and the antimicrobial is then deleted by the action of Cre protein. In some embodiments, this results in the insertion of a single *loxP* site, as well as a deletion of native DNA, as determined by the primers used to construct homologous flanking DNA and antimicrobial-containing incoming DNA.

Those of skill in the art are well aware of suitable methods for introducing 15 polynucleotide sequences into *Bacillus* cells (See e.g., Ferrari *et al.*, "Genetics," in Harwood *et al.* (ed.), *Bacillus*, Plenum Publishing Corp. [1989], pages 57-72; See also, Saunders *et al.*, *J. Bacteriol.*, 157:718-726 [1984]; Hoch *et al.*, *J. Bacteriol.*, 93:1925 -1937 [1967]; Mann *et al.*, *Current Microbiol.*, 13:131-135 [1986]; and Holubova, *Folia Microbiol.*, 30:97 [1985]; for *B. subtilis*, Chang *et al.*, *Mol. Gen. Genet.*, 168:11-115 [1979]; for *B.* 20 *megaterium*, Vorobjeva *et al.*, *FEMS Microbiol. Lett.*, 7:261-263 [1980]; for *B. amyloliquefaciens*, Smith *et al.*, *Appl. Env. Microbiol.*, 51:634 (1986); for *B. thuringiensis*, Fisher *et al.*, *Arch. Microbiol.*, 139:213-217 [1981]; and for *B. sphaericus*, McDonald, J. *Gen. Microbiol.*, 130:203 [1984]). Indeed, such methods as transformation including protoplast transformation and congression, transduction, and protoplast fusion are known 25 and suited for use in the present invention. Methods of transformation are particularly preferred to introduce a DNA construct provided by the present invention into a host cell.

In addition to commonly used methods, in some embodiments, host cells are directly transformed (*i.e.*, an intermediate cell is not used to amplify, or otherwise process, the DNA construct prior to introduction into the host cell). Introduction of the DNA 30 construct into the host cell includes those physical and chemical methods known in the art to introduce DNA into a host cell without insertion into a plasmid or vector. Such methods include, but are not limited to calcium chloride precipitation, electroporation, naked DNA, liposomes and the like. In additional embodiments, DNA constructs are co-transformed with a plasmid, without being inserted into the plasmid. In further embodiments, a 35 selective marker is deleted from the altered *Bacillus* strain by methods known in the art

- 61 -

(See, Stahl *et al.*, J. Bacteriol., 158:411-418 [1984]; and Palmeros *et al.*, Gene 247:255 - 264 [2000]).

In some embodiments, host cells are transformed with one or more DNA constructs according to the present invention to produce an altered *Bacillus* strain wherein 5 two or more genes have been inactivated in the host cell. In some embodiments, two or more genes are deleted from the host cell chromosome. In alternative embodiments, two or more genes are inactivated by insertion of a DNA construct. In some embodiments, the inactivated genes are contiguous (whether inactivated by deletion and/or insertion), while in other embodiments, they are not contiguous genes.

10 There are various assays known to those of ordinary skill in the art for detecting and measuring activity of intracellularly and extracellularly expressed polypeptides. In particular, for proteases, there are assays based on the release of acid-soluble peptides from casein or hemoglobin measured as absorbance at 280 nm or colorimetrically using the Folin method (See e.g., Bergmeyer *et al.*, "Methods of Enzymatic Analysis" vol. 5, Peptidases, Proteinases and their Inhibitors, Verlag Chemie, Weinheim [1984]). Other assays involve the 15 solubilization of chromogenic substrates (See e.g., Ward, "Proteinases," in Fogarty (ed.), Microbial Enzymes and Biotechnology, Applied Science, London, [1983], pp 251-317). Other exemplary assays include succinyl-Ala-Ala-Pro-Phe-para nitroanilide assay (SAAPFpNA) and the 2,4,6-trinitrobenzene sulfonate sodium salt assay (TNBS assay).

20 Numerous additional references known to those in the art provide suitable methods (See e.g., Wells *et al.*, Nucleic Acids Res. 11:7911-7925 [1983]; Christianson *et al.*, Anal. Biochem., 223:119 -129 [1994]; and Hsia *et al.*, Anal Biochem., 242:221-227 [1999]).

Means for determining the levels of secretion of a protein of interest in a host cell and detecting expressed proteins include the use of immunoassays with either polyclonal or 25 monoclonal antibodies specific for the protein. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), fluorescence immunoassay (FIA), and fluorescent activated cell sorting (FACS). However, other methods are known to those in the art and find use in assessing the protein of interest (See e.g., Hampton *et al.*, Serological Methods, A Laboratory Manual, APS Press, St. Paul, MN [1990]; and Maddox *et al.*, J. Exp. 30 Med., 158:1211 [1983]). In some preferred embodiments, secretion of a protein of interest is higher in the altered strain obtained using the present invention than in a corresponding unaltered host. As known in the art, the altered *Bacillus* cells produced using the present invention are maintained and grown under conditions suitable for the expression and recovery of a polypeptide of interest from cell culture (See e.g., Hardwood and Cutting (eds.) 35 Molecular Biological Methods for Bacillus, John Wiley & Sons [1990]).

B. Large Chromosomal Deletions

As indicated above, in addition to single and multiple gene deletions, the present invention provides large chromosomal deletions. In some preferred embodiments of the present invention, an indigenous chromosomal region or fragment thereof is deleted from a *Bacillus* host cell to produce an altered *Bacillus* strain. In some embodiments, the indigenous chromosomal region includes prophage regions, antimicrobial regions, (e.g., antibiotic regions), regulator regions, multi-contiguous single gene regions and/or operon regions. The coordinates delineating indigenous chromosomal regions referred to herein are specified according to the *Bacillus subtilis* strain 168 chromosome map. Numbers generally relate to the beginning of the ribosomal binding site, if present, or the end of the coding region, and generally do not include a terminator that might be present. The *Bacillus subtilis* genome of strain 168 is well known (See, Kunst *et al.*, *Nature* 390:249–256 [1997]; and Henner *et al.*, *Microbiol. Rev.*, 44:57–82 [1980]), and is comprised of one 4215 kb chromosome. However, the present invention also includes analogous sequences from any *Bacillus* strain. Particularly preferred are other *B. subtilis* strains, *B. licheniformis* strains and *B. amyloliquefaciens* strains.

In some embodiments, the indigenous chromosomal region includes prophage segments and fragments thereof. A "prophage segment" is viral DNA that has been inserted into the bacterial chromosome wherein the viral DNA is effectively indistinguishable from normal bacterial genes. The *B. subtilis* genome is comprised of numerous prophage segments; these segments are not infective. (Seaman *et al.*, *Biochem.*, 3:607–613 [1964]; and Stickler *et al.*, *Virol.*, 26:142–145 [1965]). Although any one of the *Bacillus subtilis* prophage regions may be deleted, reference is made to the following non-limiting examples.

One prophage region that is deleted in some embodiments of the present invention is a sigma K intervening "skin" element. This region is found at about 2652600 bp (*spoIVCA*) to 2700579 bp (*yqaB*) of the *B. subtilis* 168 chromosome. Using the present invention, about a 46 kb segment was deleted, corresponding to 2653562 bp to 2699604 bp of the chromosome. This element is believed to be a remnant of an ancestral temperate phage which is position within the SIGK ORF, between the genes *spoIVCB* and *spoIIIC*. However, it is not intended that the present invention be limited to any particular mechanism or mode of action involving the deleted region. The element has been shown to contain 57 open reading frames with putative ribosome binding sites (See, Takemaru *et al.*, *Microbiol.*, 141:323–327 [1995]). During spore formation in the mother cell, the skin element is excised leading to the reconstruction of the *sigK* gene.

- 63 -

Another region suitable for deletion is a prophage 7 region. This region is found at about 2701208 bp (*yrkS*) to 2749572 bp (*yraK*) of the *B. subtilis* 168 chromosome. Using the present invention, about a 48.5 kb segment was deleted, corresponding to 2701087 bp to 2749642 bp of the chromosome.

- 5 A further region is a skin + prophage 7 region. This region is found at about 2652151 bp to 2749642 bp of the *B. subtilis* 168 chromosome. Using the present invention, a segment of about 97.5 kb was deleted. This region also includes the intervening *spoIIIC* gene. The skin/prophage 7 region includes but is not limited to the following genes: *spoIVCA*-DNA recombinase, *blt* (multidrug resistance), *cypA* (cytochrome P450-like enzyme), *czcD* (cation-efflux system membrane protein), and *rapE* (response regulator aspartate phosphatase).
- 10

- 15 Yet another region is the PBSX region. This region is found at about 1319884 bp (*xkdA*) to 1347491 bp (*xlyA*) of the *B. subtilis* 168 chromosome. Using the present invention, a segment of about 29 kb was deleted, corresponding to 1319663 to 1348691 bp of the chromosome. Under normal non-induced conditions this prophage element is non-infective and is not bactericidal (except for a few sensitive strains such as W23 and S31). It is inducible with mitomycin C and activated by the SOS response and results in cell lysis with the release of phage-like particles. The phage particles contain bacterial chromosomal DNA and kill sensitive bacteria without injecting DNA. (Canosi et al., J. Gen. Virol. 39: 81–90 [1978]). This region includes the following non-limiting list of genes: *xtmA-B*; *xkdA – K and M – X*, *xre*, *xtrA*, *xpf*, *xep*, *xhIA - B* and *xlyA*.
- 20

- 25 A further region is the SP β region. This region is found at about 2150824 bp (*yodU*) to 2286246 bp (*ypqP*) of the *B. subtilis* 168 chromosome. Using the present invention, a segment of about 133.5 kb was deleted, corresponding to 2151827 to 2285246 bp of the chromosome. This element is a temperate prophage whose function has not yet been characterized. However, genes in this region include putative spore coat proteins (*yodU*, *sspC*, *yokH*), putative stress response proteins (*yorD*, *ypqQ*, *ypnP*) and other genes that have homology to genes in the spore coat protein and stress response genes such as members of the *yom* operon. Other genes in this region include: *yot*; *yos*, *yoq*, *yop*, *yon*, *yom*, *yoz*, *yol*, *yok*, *ypo*, and *ypm*.
- 30

An additional region is the prophage 1 region. This region is found at about 202098 bp (*ybbU*) to 220015 bp (*ybdE*) of the *B. subtilis* 168 chromosome. Using the present invention, a segment of about 18.0 kb was deleted, corresponding to 202112 to 220141 bp of the chromosome. Genes in this region include the *AdaA/B* operon which

- 64 -

provides an adaptive response to DNA alkylation and *ndhF* which codes for NADH dehydrogenase, subunit 5.

A further region is the prophage 2 region. This region is found at about 529069 bp (*ydcL*) to 569493 bp (*ydeJ*) of the *B. subtilis* 168 chromosome. Using the present invention, a segment of about 40.5 kb was deleted, corresponding to 529067 to 569578 bp of the chromosome. Genes in this region include *rapI/phrI* (response regulator asparate phosphatase), *sacV* (transcriptional regulator of the levensucrase) and *cspC*.

Another region is the prophage 3 region. Using the present invention, a segment of about 50.7 kb segment was deleted, corresponding to about 652000 to 664300 bp of the *B. subtilis* 168 chromosome.

Yet another region is the prophage 4 region. This region is found at about 1263017 bp (*yjcM*) to 1313627 bp (*yjoA*) of the *B. subtilis* 168 chromosome. Using the present invention, a segment of about 2.3 kb was deleted, corresponding to 1262987 to 1313692 bp of the chromosome.

An additional region is the prophage 5 region. Using the present invention a segment of about 20.8 kb segment was deleted, corresponding to about 1879200 to 1900000 bp of the *B. subtilis* 168 chromosome.

Another region is the prophage 6 region. Using the present invention a segment of about a 31.9 kb segment was deleted, corresponding to about 2046050 to 2078000 bp in the *B. subtilis* 168 chromosome.

In further embodiments, the indigenous chromosomal region includes one or more operon regions, multi-contiguous single gene regions, and/or anti-microbial regions. In some embodiments, these regions include the following:

1) The PPS operon region:

This region is found at about 1959410 bp (*ppsE*) to 1997178 bp (*ppsA*) of the *Bacillus subtilis* 168 chromosome. Using the present invention, a segment of about 38.6 kb was deleted, corresponding to about 1960409 to 1998026 bp of the chromosome. This operon region is involved in antimicrobial synthesis and encodes plipastatin synthetase;

2) The PKS operon region:

This region is found at about 1781110 bp (*pksA*) to 1857712 bp (*pksR*) of the *B. subtilis* 168 chromosome. Using the present invention, a segment of about 76.2 kb was deleted, corresponding to about 1781795 to 1857985 bp of the chromosome. This region encodes polyketide synthase and is involved in anti-microbial synthesis. (Scotti *et al.*, Gene, 130:65–71 [1993]);

- 65 -

3) The *yvfF-yveK* operon region:

This region is found at about 3513149 bp (*yvfF*) to 3528184 bp (*yveK*) of the *B. subtilis* 168 chromosome. Using the present invention, a segment of about 15.8 kb was deleted, corresponding to about 3513137 to 3528896 bp of the chromosome. This region codes for a putative polysaccharide (See, Dartois *et al.*, Seventh International Conference on *Bacillus* (1993) Institute Pasteur [1993], page 56). This region includes the following genes; *yvfA-F*, *yveK-T* and *slr*. The *slr* gene region which is found at about 3529014–3529603 bp of the *B. subtilis* 168 chromosome encompasses about a 589 bp segment. This region is the regulator region of the *yvfF-yveK* operon;

4) The DHB operon region:

This region is found at about 3279750 bp (*yukL*) to 3293206 bp (*yuiH*) of the *B. subtilis* 168 chromosome. Using the present invention, a segment of about 13.0 kb was deleted, corresponding to 3279418–3292920 bp of the chromosome. This region encodes the biosynthetic template for the catecholic siderophore 2,3-dihydroxy benzoate-glycine-threonine trimeric ester bacilibactin. (See, May *et al.*, J. Biol. Chem., 276:7209–7217 [2001]). This region includes the following genes: *yukL*, *yukM*, *dhbA – C, E* and *F*, and *yuiI-H*.

While the regions, as described above, are examples of preferred indigenous chromosomal regions to be deleted, in some embodiments of the present invention, a fragment of the region is also deleted. In some embodiments, such fragments include a range of about 1% to 99% of the indigenous chromosomal region. In other embodiments, fragments include a range of about 5% to 95% of the indigenous chromosomal region. In yet additional embodiments, fragments comprise at least 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 90%, 88%, 85%, 80%, 75%, 70%, 65%, 50%, 40%, 30%, 25%, 20% and 10% of the indigenous chromosomal region.

Further non-limiting examples of fragments of indigenous chromosomal regions to be deleted with reference to the chromosomal location in the *B. subtilis* 168 chromosome include the following:

a) for the skin region:

- i) a coordinate location of about 2666663 to 2693807, which includes *yqcC* to *yqaM*, and
- ii) a coordinate location of about 2658440 to 2659688, which includes *rapE* to *phrE*;

- 66 -

b) for the PBSX prophage region:

i) a coordinate location of about 1320043 to 1345263, which includes *xkdA* to *xkdx*, and

ii) a coordinate location of about 1326662 to 1345102, which includes *xkde* to *xkdw*;

c) for the SP β region:

i) a coordinate location of about 2149354 to 2237029, which includes *yodV* to *yonA*;

d) for the DHB region:

i) a coordinate location of about 3282879 to 3291353, which includes *dhbF* to *dhbA*;

e) for the *yvfF-yveK* region:

i) a coordinate location of about 3516549 to 3522333, which includes *yvfB* to *yveQ*,

ii) a coordinate location of about 3513181 to 3528915, which includes *yvfF* to *yveK*, and

iii) a coordinate location of about 3521233 to 3528205, which includes *yveQ* to *yveL*;

f) for the prophage 1 region:

i) a coordinate location of about 213926 to 220015, which includes *ybcO* to *ybdE*, and

ii) a coordinate location of about 214146 to 220015, which includes *ybcP* to *ybdE*;

g) for the prophage 2 region:

i) a coordinate location of about 546867 to 559005, which includes *rapI* to *cspC*; and

h) for the prophage 4 region:

i) a coordinate location of about 1263017 to 675421, which includes *yjcM* to *ydjJ*.

30

The number of fragments of indigenous chromosomal regions which are suitable for deletion are numerous, because a fragment may be comprised of only a few bps less than the identified indigenous chromosomal region. Furthermore, many of the identified indigenous chromosomal regions encompass a large number of genes. Those of skill in

- 67 -

the art are capable of easily determining which fragments of the indigenous chromosomal regions are suitable for deletion for use in a particular application.

The definition of an indigenous chromosomal region is not so strict as to exclude a number of adjacent nucleotides to the defined segment. For example, while the SP β 5 region is defined herein as located at coordinates 2150824 to 2286246 of the *B. subtilis* 168 chromosome, an indigenous chromosomal region may include a further 10 to 5000 bp, a further 100 to 4000 bp, or a further 100 to 1000 bp on either side of the region. The number of bp on either side of the region is limited by the presence of another gene not included in the indigenous chromosomal region targeted for deletion.

10 As stated above, the location of specified regions herein disclosed are in reference to the *B. subtilis* 168 chromosome. Other analogous regions from *Bacillus* strains are included in the definition of an indigenous chromosomal region. While the analogous region may be found in any *Bacillus* strain, particularly preferred analogous regions are regions found in other *Bacillus subtilis* strains, *Bacillus licheniformis* strains and *Bacillus amyloliquefaciens* strains.

15 In certain embodiments, more than one indigenous chromosomal region or fragment thereof is deleted from a *Bacillus* strain. However, the deletion of one or more indigenous chromosomal regions or fragments thereof does not deleteriously affect 20 reproductive viability of the strain which includes the deletion. In some embodiments, two indigenous chromosomal regions or fragments thereof are deleted. In additional embodiments, three indigenous chromosomal regions or fragments thereof are deleted. In yet another embodiment, four indigenous chromosomal regions or fragments thereof are deleted. In a further embodiment, five indigenous chromosomal regions or fragments thereof are deleted. In another embodiment, as many as 14 indigenous chromosomal 25 regions or fragments thereof are deleted. In some embodiments, the indigenous chromosomal regions or fragments thereof are contiguous, while in other embodiments, they are located on separate regions of the *Bacillus* chromosome.

A strain of any member of the genus *Bacillus* comprising a deleted indigenous 30 chromosomal region or fragment thereof finds use in the present invention. In some preferred embodiments, the *Bacillus* strain is selected from the group consisting of *B. subtilis* strains, *B. amyloliquefaciens* strains, *B. lentus* strains, and *B. licheniformis* strains. In some preferred embodiments, the strain is an industrial *Bacillus* strain, and most preferably an industrial *B. subtilis* strain. In a further preferred embodiment, the altered *Bacillus* strain is a protease-producing strain. In some particularly preferred embodiments,

it is a *B. subtilis* strain that has been previously engineered to include a polynucleotide encoding a protease enzyme.

As indicated above, a *Bacillus* strain in which an indigenous chromosomal region or fragment thereof has been deleted is referred to herein as "an altered *Bacillus* strain." In 5 preferred embodiments of the present invention, the altered *Bacillus* strain has an enhanced level of expression of a protein of interest (*i.e.*, the expression of the protein of interest is enhanced, compared to a corresponding unaltered *Bacillus* strain grown under the same growth conditions).

One measure of enhancement is the secretion of the protein of interest. In some 10 embodiments, production of the protein of interest is enhanced by at least 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 4.0%, 5.0%, 8.0%, 10%, 15%, 20% and 25% or more, compared to the corresponding unaltered *Bacillus* strain. In other embodiments, production of the protein of interest is enhanced by between about 0.25% to 20%; 0.5% to 15% and 1.0% to 10%, compared to the corresponding unaltered *Bacillus* strain as measured in grams of 15 protein produced per liter.

The altered *Bacillus* strains provided by the present invention comprising a deletion of an indigenous chromosomal region or fragment thereof are produced using any suitable methods, including but not limited to the following means. In one general embodiment, a DNA construct is introduced into a *Bacillus* host. The DNA construct comprises an 20 inactivating chromosomal segment, and in some embodiments, further comprises a selective marker. Preferably, the selective marker is flanked on both the 5' and 3' ends by one section of the inactivating chromosomal segment.

In some embodiments, the inactivating chromosomal segment, while preferably having 100% sequence identity to the immediate upstream and downstream nucleotides 25 of an indigenous chromosomal region to be deleted (or a fragment of said region), has between about 70 to 100%, about 80 to 100%, about 90 to 100%, and about 95 to 100% sequence identity to the upstream and downstream nucleotides of the indigenous chromosomal region. Each section of the inactivating chromosomal segment must include sufficient 5' and 3' flanking sequences of the indigenous chromosomal region to provide 30 for homologous recombination with the indigenous chromosomal region in the unaltered host.

In some embodiments, each section of the inactivating chromosomal segment comprises about 50 to 10,000 base pairs (bp). However, lower or higher bp sections find use in the present invention. Preferably, each section is about 50 to 5000 bp, about 100

- 69 -

to 5000 bp, about 100 to 3000 bp; 100 to 2000 bp; about 100 to 1000 bp; about 200 to 4000 bp, about 400 to 3000 bp, about 500 to 2000 bp, and also about 800 to 1500 bp.

In some embodiments, a DNA construct comprising a selective marker and an inactivating chromosomal segment is assembled *in vitro*, followed by direct cloning of said construct into a competent *Bacillus* host, such that the DNA construct becomes integrated into the *Bacillus* chromosome. For example, PCR fusion and/or ligation are suitable for assembling a DNA construct *in vitro*. In some embodiments, the DNA construct is a non-plasmid construct, while in other embodiments, it is incorporated into a vector (*i.e.*, a plasmid). In some embodiments, a circular plasmid is used, and the circular plasmid is cut using an appropriate restriction enzyme (*i.e.*, one that does not disrupt the DNA construct). Thus, linear plasmids find use in the present invention (See e.g., Figure 1; and Perego, "Integrational Vectors for Genetic Manipulation in *Bacillus subtilis*," in *Bacillus subtilis and other Gram-Positive Bacteria*, Sonenshein. et al., Eds., Am. Soc. Microbiol., Washington, DC [1993]).

In some embodiments, a DNA construct or vector, preferably a plasmid including an inactivating chromosomal segment includes a sufficient amount of the 5' and 3' flanking sequences (seq) of the indigenous chromosomal segment or fragment thereof to provide for homologous recombination with the indigenous chromosomal region or fragment thereof in the unaltered host. In another embodiment, the DNA construct includes restriction sites engineered at upstream and downstream ends of the construct. Non-limiting examples of DNA constructs useful according to the invention and identified according to the coordinate location include:

1. A DNA construct for deleting a PBSX region: [5' flanking seq 1318874 - 1319860 bp which includes the end of *yjqB* and the entire *yjpC* including the ribosome binding site (RBS)] -marker gene - [3' flanking seq 1348691 - 1349656 bp which includes a terminator and upstream section of the *pit*].
2. A DNA construct for deleting a prophage 1 region: [5' flanking seq 201248 - 202112 bp which contains the entire *glmS* including the RBS and terminator and the *ybbU* RBS] - marker gene - [3' flanking seq 220141 - 221195 bp which includes the entire *ybgd* including the RBS].
3. A DNA construct for deleting a prophage 2 region: [5' flanking seq 527925 - 529067 bp which contains the end of *ydcK*, the entire tRNAs as follows: trnS-Asn, trnS-Ser, trnS-Glu, trnS-Gln, trnS-Lys, trnS-Leu1 and trnS-leu2] -marker gene - [3' flanking seq 569578 - 571062 bp which contains the entire *ydeK* and upstream part of *ydeL*].

- 70 -

4. A DNA construct for deleting a prophage 4 region: [5' flanking seq 1263127 - 1264270 bp which includes part of *yjCM*] - marker gene - [3' flanking seq 1313660 - 1314583 bp which contains part of *yjOB* including the RBS].

5. A DNA construct for deleting a *yvfF-yvεK* region: [5' flanking seq 3512061 - 3513161 bp which includes part of *sigL*, the entire *yvfG* and the start of *yvfF*] - marker gene - [3' flanking seq 3528896 - 3529810 bp which includes the entire *sir* and the start of *pnbA*].

10. 6. A DNA construct for deleting a DHB operon region: [5' flanking seq 3278457 - 3280255 which includes the end of *ald* including the terminator, the entire *yuxI* including the RBS, the entire *yukJ* including the RBS and terminator and the end of *yukL*] - marker gene - [3' flanking seq 3292919 - 3294076 which includes the end of *yuiH* including the RBS, the entire *yuiG* including the RBS and terminator and the upstream end of *yuiF* including the terminator.

15. Whether the DNA construct is incorporated into a vector or used without the presence of plasmid DNA, it is introduced into a microorganism, preferably an *E. coli* cell or a competent *Bacillus* cell.

20. Methods for introducing DNA into *Bacillus* cells involving plasmid constructs and transformation of plasmids into *E. coli* are well known. The plasmids are subsequently isolated from *E. coli* and transformed into *Bacillus*. However, it is not essential to use intervening microorganisms such as *E. coli*, and in some embodiments, a DNA construct or vector is directly introduced into a *Bacillus* host.

In a preferred embodiment, the host cell is a *Bacillus* sp. (See e.g., U.S. Patent No. 5,264,366, U.S. Patent No. 4,760,025, and RE 34,6060). In some embodiments, the *Bacillus* strain of interest is an alkalophilic *Bacillus*. Numerous alkalophilic *Bacillus* strains are known (See e.g., U.S. Patent 5,217,878; and Aunstrup et al., Proc IV IFS: Ferment. Tech. Today, 299-305 [1972]). Another type of *Bacillus* strain of particular interest is a cell of an industrial *Bacillus* strain. Examples of industrial *Bacillus* strains include, but are not limited to *B. licheniformis*, *B. lentus*, *B. subtilis*, and *B. amyloliquefaciens*. In additional embodiments, the *Bacillus* host strain is selected from the group consisting of *B. licheniformis*, *B. subtilis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. thuringiensis*, *B. clausii*, and *B. megaterium*. In particularly preferred embodiments, *B. subtilis* cells are used.

35. In some embodiments, the industrial host strains are selected from the group consisting of non-recombinant strains of *Bacillus* sp., mutants of a naturally-occurring *Bacillus* strain, and recombinant *Bacillus* host strains. Preferably, the host strain is a

- 71 -

recombinant host strain, wherein a polynucleotide encoding a polypeptide of interest has been previously introduced into the host. A further preferred host strain is a *Bacillus subtilis* host strain, and particularly a recombinant *Bacillus subtilis* host strain. Numerous *B. subtilis* strains are known and suitable for use in the present invention (See e.g., 1A6 5 (ATCC 39085), 168 (1A01), SB19, W23, Ts85, B637, PB1753 through PB1758, PB3360, JH642, 1A243 (ATCC 39,087), ATCC 21332, ATCC 6051, MI113, DE100 (ATCC 39,094), GX4931, PBT 110, and PEP 211strain; Hoch *et al.*, Genetics, 73:215–228 [1973]; U.S. Patent No. 4,450,235; U.S. Patent No. 4,302,544; EP 0134048; Palva *et al.*, Gene, 19:81-10 87 [1982]; Fahnstock and Fischer, J. Bacteriol., (1986) 165:796 - 804 [1986]; and Wang *et al.*, Gene 69:39-47 [1988]). Of particular interest as expression hosts are industrial protease-producing *Bacillus* strains. By using these strains, the high efficiency seen for production of the protease is further enhanced by the altered *Bacillus* strain of the present invention.

Industrial protease producing *Bacillus* strains provide particularly preferred 15 expression hosts. In some preferred embodiments, use of these strains in the present invention provides further enhancements in efficiency and protease production. As indicated above, there are two general types of proteases are typically secreted by *Bacillus sp.*, namely neutral (or "metalloproteases") and alkaline (or "serine") proteases. Also as indicated above, subtilisin is a preferred serine protease for use in the present 20 invention. A wide variety of *Bacillus* subtilisins have been identified and sequenced, for example, subtilisin 168, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin 147 and subtilisin 309 (See e.g., EP 414279 B; WO 89/06279; and Stahl *et al.*, J. Bacteriol., 159:811–818 [1984]). In some embodiments of the present invention, the *Bacillus* host strains produce mutant (e.g., variant) proteases. Numerous references provide examples 25 of variant proteases and reference (See e.g., WO 99/20770; WO 99/20726; WO 99/20769; WO 89/06279; RE 34,606; U.S. Patent No. 4,914,031; U.S. Patent No. 4,980,288; U.S. Patent No. 5,208,158; U.S. Patent No. 5,310,675; U.S. Patent No. 5,336,611; U.S. Patent No. 5,399,283; U.S. Patent No. 5,441,882; U.S. Patent No. 5,482,849; U.S. Patent No. 5,631,217; U.S. Patent No. 5,665,587; U.S. Patent No. 5,700,676; U.S. Patent No. 5,741,694; U.S. Patent No. 5,858,757; U.S. Patent No. 5,880,080; U.S. Patent No. 6,197,567; and U.S. Patent No. 6,218,165.

In yet another embodiment, a preferred *Bacillus* host is a *Bacillus* sp. that includes a mutation or deletion in at least one of the following genes, *degU*, *degS*, *degR* and *degQ*. Preferably the mutation is in a *degU* gene, and more preferably the mutation is 35 *degU(Hy)32*. (See, Msadek *et al.*, J. Bacteriol., 172:824-834 [1990]; and Olmos *et al.*,

- 72 -

Mol. Gen. Genet., 253:562-567 [1997]). A most preferred host strain is a *Bacillus subtilis* carrying a *degU32(Hy)* mutation. In a further embodiment, the *Bacillus* host comprises a mutation or deletion in *scoC4*, (See, Caldwell *et al.*, J. Bacteriol., 183:7329-7340 [2001]); *spolIIE* (See, Arigoni *et al.*, Mol. Microbiol., 31:1407-1415 [1999]); *oppA* or other genes of 5 the *opp* operon (See, Perego *et al.*, Mol. Microbiol., 5:173-185 [1991]). Indeed, it is contemplated that any mutation in the *opp* operon that causes the same phenotype as a mutation in the *oppA* gene will find use in some embodiments of the altered *Bacillus* strain of the present invention. In some embodiments, these mutations occur alone, while in other embodiments, combinations of mutations are present. In some embodiments, an 10 altered *Bacillus* of the invention is obtained from a *Bacillus* host strain that already includes a mutation in one or more of the above-mentioned genes. In alternate embodiments, an altered *Bacillus* of the invention is further engineered to include mutation 15 in one or more of the above-mentioned genes.

In some embodiments, two or more DNA constructs are introduced into a *Bacillus* host cell, resulting in the deletion of two or more indigenous chromosomal regions in an 20 altered *Bacillus*. In some embodiments, these regions are contiguous, (e.g., the skin plus prophage 7 region), while in other embodiments, the regions are separated (e.g., the PBSX region and the PKS region; the skin region and the DHB region; or the PKS region, the SP β region and the *yvfF-yveK* region).

Those of skill in the art are well aware of suitable methods for introducing 25 polynucleotide sequences into bacterial (e.g., *E. coli* and *Bacillus*) cells (See e.g., Ferrari *et al.*, "Genetics," in Harwood *et al.* (ed.), *Bacillus*, Plenum Publishing Corp. [1989], pages 57-72; See also, Saunders *et al.*, J. Bacteriol., 157:718-726 [1984]; Hoch *et al.*, J. Bacteriol., 93:1925 -1937 [1967]; Mann *et al.*, Current Microbiol., 13:131-135 [1986]; and 30 Holubova, Folia Microbiol., 30:97 [1985]; for *B. subtilis*, Chang *et al.*, Mol. Gen. Genet., 168:11-115 [1979]; for *B. megaterium*, Vorobjeva *et al.*, FEMS Microbiol. Lett., 7:261-263 [1980]; for *B. amyloliquefaciens*, Smith *et al.*, Appl. Env. Microbiol., 51:634 (1986); for *B. thuringiensis*, Fisher *et al.*, Arch. Microbiol., 139:213-217 [1981]; and for *B. sphaericus*, McDonald, J. Gen. Microbiol., 130:203 [1984]). Indeed, such methods as transformation 35 including protoplast transformation and congression, transduction, and protoplast fusion are known and suited for use in the present invention. Methods of transformation are particularly preferred to introduce a DNA construct provided by the present invention into a host cell.

In addition to commonly used methods, in some embodiments, host cells are 40 directly transformed (i.e., an intermediate cell is not used to amplify, or otherwise process,

- 73 -

the DNA construct prior to introduction into the host cell). Introduction of the DNA construct into the host cell includes those physical and chemical methods known in the art to introduce DNA into a host cell, without insertion into a plasmid or vector. Such methods include but are not limited to calcium chloride precipitation, electroporation, naked DNA, liposomes and the like. In additional embodiments, DNA constructs are co-transformed with a plasmid without being inserted into the plasmid. In a further embodiments, a selective marker is deleted or substantially excised from the altered *Bacillus* strain by methods known in the art (See, Stahl *et al.*, J. Bacteriol., 158:411-418 [1984]; and the conservative site-specific recombination [CSSR] method of Palmeros *et al.*, described in Palmeros *et al.*, Gene 247:255 -264 [2000]). In some preferred embodiments, resolution of the vector from a host chromosome leaves the flanking regions in the chromosome while removing the indigenous chromosomal region.

In some embodiments, host cells are transformed with one or more DNA constructs according to the present invention to produce an altered *Bacillus* strain wherein two or more genes have been inactivated in the host cell. In some embodiments, two or more genes are deleted from the host cell chromosome. In alternative embodiments, two or more genes are inactivated by insertion of a DNA construct. In some embodiments, the inactivated genes are contiguous (whether inactivated by deletion and/or insertion), while in other embodiments, they are not contiguous genes.

As indicated above, there are various assays known to those of ordinary skill in the art for detecting and measuring activity of intracellularly and extracellularly expressed polypeptides. In particular, for proteases, there are assays based on the release of acid-soluble peptides from casein or hemoglobin measured as absorbance at 280 nm or colorimetrically using the Folin method (See e.g., Bergmeyer *et al.*, "Methods of Enzymatic Analysis" vol. 5, Peptidases, Proteinases and their Inhibitors, Verlag Chemie, Weinheim [1984]). Other assays involve the solubilization of chromogenic substrates (See e.g., Ward, "Proteinases," in Fogarty (ed.), Microbial Enzymes and Biotechnology, Applied Science, London, [1983], pp 251-317). Other exemplary assays include succinyl-Ala-Ala-Pro-Phe-para nitroanilide assay (SAAPFpNA) and the 2,4,6-trinitrobenzene sulfonate sodium salt assay (TNBS assay). Numerous additional references known to those in the art provide suitable methods (See e.g., Wells *et al.*, Nucleic Acids Res. 11:7911-7925 [1983]; Christianson *et al.*, Anal. Biochem., 223:119 -129 [1994]; and Hsia *et al.*, Anal Biochem., 242:221-227 [1999]).

Also as indicated above, means for determining the levels of secretion of a protein of interest in a host cell and detecting expressed proteins include the use of immunoassays with

- 74 -

either polyclonal or monoclonal antibodies specific for the protein. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), fluorescence immunoassay (FIA), and fluorescent activated cell sorting (FACS). However, other methods are known to those in the art and find use in assessing the protein of interest (See e.g., 5 Hampton et al., Serological Methods, A Laboratory Manual, APS Press, St. Paul, MN [1990]; and Maddox et al., J. Exp. Med., 158:1211 [1983]). In some preferred embodiments, secretion of a protein of interest is higher in the altered strain obtained using the present invention than in a corresponding unaltered host. As known in the art, the altered *Bacillus* cells produced using the present invention are maintained and grown under conditions 10 suitable for the expression and recovery of a polypeptide of interest from cell culture (See e.g., Hardwood and Cutting (eds.) Molecular Biological Methods for Bacillus, John Wiley & Sons [1990]).

The manner and method of carrying out the present invention may be more fully understood by those of skill in the art by reference to the following examples, which examples 15 are not intended in any manner to limit the scope of the present invention or of the claims directed thereto.

EXPERIMENTAL

The following Examples are provided in order to demonstrate and further illustrate 20 certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); H₂O (water); dH₂O (deionized water); (HCl (hydrochloric acid); aa (amino acid); bp (base pair); kb (kilobase pair); 25 kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); U (units); V (volts); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); MgCl₂ (magnesium chloride); NaCl (sodium chloride); OD₂₈₀ (optical density at 280 nm); OD₆₀₀ (optical density at 600 30 nm); PAGE (polyacrylamide gel electrophoresis); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); PEG (polyethylene glycol); PCR (polymerase chain reaction); RT-PCR (reverse transcription PCR); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume); LA medium (per liter: Difco Tryptone Peptone 20g, Difco Yeast Extract 10g, EM 35 Science NaCl 1g, EM Science Agar 17.5g, dH₂O to 1L); ATCC (American Type Culture

- 75 -

Collection, Rockville, MD); Clontech (CLONTECH Laboratories, Palo Alto, CA); Difco (Difco Laboratories, Detroit, MI); GIBCO BRL or Gibco BRL (Life Technologies, Inc., Gaithersburg, MD); Invitrogen (Invitrogen Corp., San Diego, CA); NEB (New England Biolabs, Beverly, MA); Sigma (Sigma Chemical Co., St. Louis, MO); Takara (Takara Bio Inc. Otsu, Japan); Roche Diagnostics and Roche (Roche Diagnostics, a division of F. Hoffmann La Roche, Ltd., Basel, Switzerland); EM Science (EM Science, Gibbstown, NJ); Qiagen (Qiagen, Inc., Valencia, CA); Stratagene (Stratagene Cloning Systems, La Jolla, CA); Affymetrix (Affymetrix, Santa Clara, California).

10

EXAMPLE 1

Creation of Deletion Strains

This Example describes "Method 1," which is also depicted in Figure 1. In this method, *E. coli* was used to produce a pJM102 plasmid vector carrying the DNA construct to be transformed into *Bacillus* strains. (See, Perego, *supra*). Regions immediately flanking the 5' and 3' ends of the deletion site were PCR amplified. PCR primers were designed to be approximately 37 base pairs in length, including 31 base pairs homologous to the *Bacillus subtilis* chromosome and a 6 base pair restriction enzyme site located 6 base pairs from the 5' end of the primer. Primers were designed to engineer unique restriction sites at the upstream and downstream ends of the construct and a *Bam*HI site between the two fragments for use in cloning. Primers for the antimicrobial markers contained *Bam*HI sites at both ends of the fragment. Where possible, PCR primers were designed to remove promoters of deleted indigenous chromosomal regions, but to leave all terminators in the immediate area. The primary source of chromosome sequence, gene localization, and promoter and terminator information was obtained from Kunst et al., (1997) *supra* and also obtainable from the SubtiList World Wide Web Server known to those in the art (See e.g., Moszer et al., *supra*). Numerous deletions have been made using the present invention. A list of primer sequences from deletions created by this method is provided in Table 1. Reference is also made to Figure 2 for an explanation of the primer naming system.

- 76 -

Table 1. Primers

Primer Name	Restriction Enzyme Engineered Into Primer	Primer Sequence	SEQ ID NO
PBSX-UF	XbaI	CTACATTCTAGACGATTTGTTGATCGATATGTGGAAGC	60
PBSX-UR	BamHI	GGCTGAGGATCCATTCCCTCAGCCCAGAAGAGAACCTA	61
PBSX-DF	BamHI	TCCCTCGGATCCGAAATAGGTTCTGCTTATTGTATTG	62
PBSX-DR	SacI	AGCGTTGAGCTCGGCCATGCCATTATATTGGCTGCTG	63
Pphage 1-	EcoRI	GTGACGGAATTCACGTGCGTCTTATATTGCTGAGCTT	64
Pphage 1-	BamHI	CGTTTGATCCAAAACACCCCTTAGATAATCTTAT	65
Pphage 1-	BamHI	ATCAAAGGATCCGCTATGCTCCAAATGTACACCTTCCGT	66
Pphage 1-	PstI	ATATTCTGCAGGCTGATATAATAACTGTGTGTTCC	67
Pphage 2-	SacI	CATCTTGAATTCAAAGGGTACAAGCACAGAGACAGAG	68
Pphage 2-	BamHI	TGACTTGGATCCGGTAAGTGGCAGTTGTGGCAGT	69
Pphage 2-	BamHI	TAGATAGGATCCTATTGAAAATCTGTTAAGAAGAGGA	70
Pphage 2-	PstI	CTGATTCTGCAGGAGTGTGTTGAAGGAAGCTTCATT	71
Pphage 4-	KpnI	CTCCGCGGTACCGTCACGAATGCGCCTTATTCTAT	72
Pphage 4-	BamHI	TCGCTGGATCCTTGGCGCCGTGGAATGATTTGTCC	73
Pphage 4-	BamHI	GCAATGGGATCCTATATCAACGGTTATGAATTCAA	74
Pphage 4-	PstI	CCAGAACTGCAGGAGCGAGGCCTCGCTGCCTGAAA	75
PPS-UF	SacI	GACAAGGAGCTCATGAAAAAAAGCATAAAGCTTATGTTGC	76
PPS-UR	BamHI	GACAAGGGATCCCGGCATGTCGTTTACTTAATTTC	77
PPS-DF	BamHI	GACAAGGGATCCTGCCGCTTACCGGAAACGGA	78
PPS-DR	XbaI	GACAAGTCTAGATTATGTTGTGCAGTATTACTTG	79
SP β -UF	SacI	ACTGATGAGCTCTGCCCTAACAGCAAACAGCAGAAC	80
SP β -UR	BamHI	ACGAATGGATCCATCATAAAGCCGCAGCAGATTAAATAT	81
SP β -DF	BamHI	ACTGATGGATCCATCTCGATAAAATATGAAAGTGGC	82
SP β -DR	XbaI	ACTGATTCTAGAGCCTTTCTCTGATGCAATTCTTC	83
PKS-UF	XbaI	GAGCCTCTAGAGCCCATTGAATCATTGTT	84
PKS-UR	BamHI	GAGCCGGATCCTAAGGATGCGTTGTGTCT	85
PKS-DF	BamHI	GAGCCGGATCCATTGGGGTTCTCAAAAAAA	86
PKS-DR	SacI	GAGCCGAGCTCATGCAAATGGAAAAATTGAT	87
Skin-UF	XbaI	GAAGTTCTAGAGATTGTAATTACAAAAGGGGGTG	88
Skin-UR	BamHI	GAAGTGGATCCTTCACCGATCATAAAAGCCC	89
Skin-DF	BamHI	TGAAAGGATCCATTTCATTGATTGTTAAGTC	90
Skin-DR	SacI	GAAGTTAGAGCTGGGGGGCATAAATTCCCG	91
Phleo-UF	BamHI	GCTTATGGATCCGATACAAGAGAGGTCTCTCG	92
Phleo-DR	BamHI	GCTTATGGATCCCTGTATGGCGCATTACAG	93
Spec-UF	BamHI	ACTGATGGATCCATCGATTTCGTTGTAATACATG	94
Spec-DR	BamHI	ACTGATGGATCCCATATGCAAGGGTTATTGTTTC	95
CssS-UF	XbaI	GCACGTTCTAGACCACCGTCCCTGTGTTATCCAC	96
CssS-UR	BamHI	AGGAAGGGATCCAGAGCGAGGAAGATGTAGGATGATC	97
CssS-DF	BamHI	TGACAAGGATCCTGTATCATAACCGCATAGCAGTGC	98

- 77 -

CssS-DR	SacI	TTCCCGCAGCTCGCGAGAGCTTCAGACTCCGTAGA	99
SBO-	XbaI	GAGCCTCTAGATCAGCGATTGACCGGGCGC	100
SBO-	BamHI	TTATCTGGATCCTGATGAGCAATGATGGTAAGATAGA	101
SBO-	BamHI	GGGTAAGGATCC CCCAAAAGGGCATAGTCATTCTACT	102
SBO-	Asp718	GAGATCGGTACC CTTTGGGCCATATCGTGGATTTC	103
PhrC-UF	HindIII	GAGCC AAGCTT CATTGACAGCAACCAGGCAGATCTC	104
PhrC-DF	PstI	GCTTATAAGCTTGATACAAGAGAGGTCTCTCG	105
PhrC-UR	PstI	GCTTATAAGCTTCTGTATGGCGCATTAACG	106
PhrC-DR	SacI	GAGCCGAGCTC CATGCCGATGAAGTCATCGTCGAGC	107
PhrC-UF-	HindIII	CGTGAA AAGCTT TCGCGGGATGTATGAATTGATAAG	108
PhrC-DR-	SacI	TGTAGGGAGCTC GATGCCACAAATGTCGGTACAACG	109

The restriction sites are designated as follows: *Xba*I is TCTAGA; *Bam*HI is GGATCC; *Sac*I is GAGCTC; *Asp*718 is GGTACC; *Pst*I is CTGCAG and *Hind*III is AAGCTT. Also prophage is designated as "Pphage."

5

In this method, 100 µL PCR reactions carried out in 150µL Eppendorf tubes containing 84µL water, 10µL PCR buffer, 1µL of each primer (*i.e.*, PKS-UF and PKS-UR), 2µL of dNTPs, 1 µL of wild type *Bacillus* chromosomal DNA template, and 1µL of polymerase. DNA polymerases used included *Taq* Plus Precision polymerase and 10 Herculase (Stratagene). Reactions were carried out in a Hybaid PCRExpress thermocycler using the following program. The samples were first heated at 94°C for 5 minutes, then cooled to a 50° hold. Polymerase was added at this point. Twenty-five cycles of amplification consisted of 1 minute at 95°C, 1 minute at 50°C and 1 minute at 15 72°C. A final 10 minutes at 72°C ensured complete elongation. Samples were held at 4°C for analysis.

After completion of the PCR, 10µL of each reaction were run on an Invitrogen 1.2% agarose E-gel at 60 volts for 30 minutes to check for the presence of a band at the correct size. All the gel electrophoresis methods described herein used these conditions. If a band was present, the remainder of the reaction tube was purified using 20 the Qiagen Qiaquick® PCR purification kit according to the manufacturer's instructions, then cut with the appropriate restriction enzyme pair. Digests were performed at 37°C for 1 hour as a 20 µL reaction consisting of 9µL of water, 2µL of 10xBSA, 2µL of an appropriate NEB restriction buffer (according to the 2000-01 NEB Catalog and Technical Reference), 5 µL of template, and 1µL of each restriction enzyme. For 25 example, the PBSX upstream fragment and CssS upstream fragments were cut with *Xba*I and *Bam*HI in NEB (New England BioLabs) restriction buffer B. The digested fragments were purified by gel electrophoresis and extraction using the Qiagen

- 78 -

Qiaquick gel extraction kit following the manufacturer's instructions. Figures 5 and 6 provide gels showing the results for various deletions.

Ligation of the fragments into a plasmid vector was done in two steps, using either the Takara ligation kit following the manufacturer's instructions or T4 DNA ligase (Reaction 5 contents: 5 µL each insert fragment, 1µL cut pJM102 plasmid, 3 µL T4 DNA ligase buffer, and 1 µL T4 DNA ligase). First, the cut upstream and downstream fragments were ligated overnight at 15°C into unique restriction sites in the pJM102 plasmid polylinker, connecting at the common *Bam*HI site to re-form a circular plasmid. The pJM102 plasmid was cut with the unique restriction enzyme sites appropriate for each deletion (See, Table 2; for *cssS*, 10 *Xba*I and *Sac*I were used) and purified as described above prior to ligation. This re-circularized plasmid was transformed into Invitrogen's "Top Ten" *E. coli* cells, using the manufacturers One Shot transformation protocol.

Transformants were selected on Luria-Bertani broth solidified with 1.5% agar (LA) plus 50 ppm carbanicillin containing X-gal for blue-white screening. Clones were picked 15 and grown overnight at 37°C in 5mL of Luria Bertani broth (LB) plus 50 ppm carbanicillin and plasmids were isolated using Qiagen's Qiaquick Mini-Prep kit. Restriction analysis confirmed the presence of the insert by cutting with the restriction sites at each end of the insert to drop an approximately 2 kb band out of the plasmid. Confirmed plasmids with the insert were cut with *Bam*HI to linearize them in digestion reactions as described above 20 (with an additional 1 µL of water in place of a second restriction enzyme), treated with 1 µL calf intestinal and shrimp phosphatases for 1 hour at 37°C to prevent re-circularization, and ligated to the antimicrobial resistance marker as listed in Table 2. Antimicrobial markers were cut with *Bam*HI and cleaned using the Qiagen Gel Extraction Kit following manufacturer's instructions prior to ligation. This plasmid was cloned into *E. coli* as 25 before, using 5 ppm phleomycin (phl) or 100 ppm spectinomycin (spc) as appropriate for selection. Confirmation of marker insertion in isolated plasmids was done as described above by restriction analysis with *Bam*HI. Prior to transformation into *B. subtilis*, the plasmid was linearized with *Sal*I to ensure a double crossover event.

30 **Table 2. Unique Restriction Enzyme Pairs Used in Deletion Constructs**

Deletion Name	Unique Restriction Enzyme Pair	Antimicrobial Marker
<i>Sbo</i>	<i>Xba</i> - <i>Asp718</i>	<i>spc</i>
<i>Slr</i>	<i>Xba</i> – <i>Sac</i> I	<i>phleo</i>

- 79 -

<i>YbcO</i>	XbaI - SacI	spc
<i>Csn</i>	XbaI - Sall	phleo
<i>PBSX</i>	XbaI-SacI	phl
<i>PKS</i>	XbaI-SacI	phl
<i>SPβ</i>	XbaI-SacI	spec
<i>PPS</i>	XbaI-SacI	spec
<i>Skin</i>	XbaI-SacI	phl

EXAMPLE 2**Creation of DNA Constructs Using PCR Fusion to Bypass *E. coli***

5 This Example describes "Method 2," which is also depicted in Figure 3. Upstream and downstream fragments were amplified as in Method 1, except the primers were designed with 25 bp "tails" complementary to the antimicrobial marker's primer sequences. A "tail" is defined herein as base pairs on the 5' end of a primer that are not homologous to 10 the sequence being directly amplified, but are complementary to another sequence of DNA. Similarly, the primers for amplifying the antimicrobial contain "tails" that are complementary to the fragments' primers. For any given deletion, the DeletionX-UFFfus and DeletionX-URfus are direct complements of one another. This is also true for the DF-fus and DR-fus primer sets. In addition, in some embodiments, these primers contain 15 restriction enzyme sites similar to those used in Method 1 for use in creating a plasmid vector (See, Table 3 and U.S. Patent No. 5,023,171). Table 3 provides a list of primers useful for creation of deletion constructs by PCR fusion. Table 4 provides an additional list of primers useful for creation of deletion constructs by PCR fusion. However, in this Table, all deletion constructs would include the phleo^R marker.

20

Table 3. Primers

Primer name	Restriction enzyme engineered into primer	Sequence	SEQ ID. NO.
DHB-UF	XbaI	CGAGAACATGAAACAGGATGAATCATCTGTGGCGGG	110
DHB-UFFfus-phleo	BamHI	CGACTGTCCAGCCGCTCGGCACATCGGATCCGCTTA CCGAAAGCCAGACTCAGCAA	111
DHB-URfus-phleo	BamHI	TTGCTGAGTCTGGCTTCGGTAAGCGGATCCGATGTG CCGAGCGGCTGGACAGTCG	112

- 80 -

DHB-DFfus-phleo	BamHI	CGTTAATGCGCCATGACAGCCATGAGGATCCCACAA GCCCGCACGCCTGCCACAC	113
DHB-DRfus-phleo	BamHI	GTGTGGCAAGGCGTGCAGGGCTTGAGGATCCTCATG GCTGTCATGGCGCATTAAACG	114
DHB-DR	SacI	GACTTCGTCGACGAGTGCAGGACGGCCAGCATCACCA	115
DHB-UF-nested	XbaI	GGCATATCTAGAGACATGAAGCAGGGAAACAGATG	116
DHB-DR-nested	SacI	GGTGCAGGAGCTGACAGTATCACAGCCAGCGCTG	117
YvfF-yveK-UF	XbaI	AAGCGTTCTAGACTGCAGGATGCAGATCGATCTCGGG	118
YvfF-yveK-UF-phleo	BamHI	AACCTCCGCTCACATGTGAGCAGGGATCC GCTTACCGAAAGCCAGACTCAGCAA	119
YvfF-yveK-UR-phleo	BamHI	TTGCTGAGTCTGGCTTCGGTAAGCGGATCC CCTGCTCACATGTGAGCGGAAGGTT	120
YvfF-yveK-DF-phleo	BamHI	CGTTAATGCGCCATGACAGCCATGAGGATCC GCCCTCAGCCTTCCGCGGCTGGCT	121
YvfF-yveK-DR-phleo	BamHI	AGCCAGCCGCGGAAGGCTGAAGCGGATCC TCATGGCTGTACGGCGCATTAAACG	122
YvfF-yveK-DR	PstI	CAAGCACTGCAGCCCACACTTCAGGCGGCTCAGGTC	123
YvfF-yveK-UF-	XbaI	GAGATATCTAGAATGGTATGAAGCGGAATTCCCG	124
YvfF-yveK-DR-	KpnI	ATAAACGGTACCCCCCTATAGATGCGAACGTTAGCCC	125
Prophage7-UF	EcoRI	AAGGAGGAATTCCATCTGAGGTATACAAACAGTCAT	126
Prophage 7-UF-	BamHI	TCTCCGAGAAAGACAGGCAGGATCGGATCC	127
Prophage 7-UR-	BamHI	TTGCTGAGTCTGGCTTCGGTAAGCGGATCC	128
Skin+prophage7-	Asp718	AAGGACGGTACCGGCTCATTACCTCTTTCAAGGGT	129
Skin+pro7-UF-phleo	BamHI	ACCAAAGCCGGACTCCCCCGCAGAGGGATCC GCTTACCGAAAGCCAGACTCAGCAA	130
Skin+pro7-UR-phleo	BamHI	TTGCTGAGTCTGGCTTCGGTAAGCGGATCC TCTCGGGGGAGTCCGGCTTGGT	131
Skin+pro7-DF-phleo	BamHI	CGTTAATGCGCCATGACAGCCATGA GGATCCCACGGGGTACACAATGTACCATA	132
Skin+pro7-DR-phleo	BamHI	TATGGTACATTGTGACCCGTATGGGATCC TCATGGCTGTACGGCGCATTAAACG	133
Skin+pro7-DR	PstI	GTCAACCTGCAGAGCGGCCAGGTACAAGTTGGGGA	134
Skin+pro7-UF-	SacI	GGATCAGAGCTCGCTTGTCCCTCTGGGAACAGCCGG	135
Skin+pro7-DR-	PstI	TATATGCTGCAGGGCTCAGACGGTACCGTTGTTCCCT	136

The restriction sites are designated as follows: *Xba*I is TCTAGA; *Bam*HI is GGATCC; *Sac*I is GAGCTC; *Asp*718 is GGTACC; *Pst*I is CTGCAG and *Hind*III is AAGCTT.

- 81 -

Table 4. Additional Primers Used to Create Deletion Constructs by PCR Fusion*.

Primer Name	Restriction Enzyme Engineered Into Primer	Sequence	SEQ ID NO:
Slr-UF	XbaI	CTGAACCTAGACCTTCACCAGGCACAGAGGAGGTGA	137
Slr-Uffus	BamHI	GCCAATAAGTTCTTTAGAGAACAGGATCC GCTTACCGAAAGCCAGACTCAGCAA	138
Slr-Urfus	BamHI	TTGCTGAGTCTGGCTTCGGTAAGCGGATCCTGTTCT AAAGAGAACTTATTGGC	139
Slr-Dffus	BamHI	CGTTAATGCCCATGACAGCCATGAGGATCC GGGCTAACGTTCGCATCTATAGGGG	140
Slr-Drfus	BamHI	CCCCTATAGATGCGAACGTTAGCCC GGATCC TCATGGCTGTCATGGCGCATTAAACG	141
Slr-DR	SacI	TGAGACGAGCTCGATGCATAGGCACGGCAGGGCGCC	142
Slr-UF- nested	XbaI	CGAAATTCTAGATCCCGCGATTCCGCCCTTGTGG	143
Slr-DR-nested	SacI	TTCCAAGAGCTCGCGGAATACCGGAAGCAGCCCC	144
YbcO-UF	XbaI	CAATTCTCTAGAGCGGTGGCGCAGGTATAGGAGGGG	145
YbcO-UF	BamHI	GAAAAGAAACCAAAAAGAATGGGAAGGATCC GCTTACCGAAAGCCAGACTCAGCAA	146
YbcO-UR	BamHI	TTGCTGAGTCTGGCTTCGGTAAGCGGATCC TTCCCATTTCTTTGGTTCTTT	147
YbcO-DF	BamHI	CGTTAATGCCCATGACAGCCATGAGGATCC GCTATTTAACATTTGAGAATAGGGGA	148
YbcO-DR	BamHI	TCCCTATTCTCAAATGTTAATAGCGGATCC TCATGGCTGTCATGGCGCATTAAACG	149
YbcO-DR	SacI	CAGGCGGAGCTCCCATTTATGACGTGCTCCCTAACG	150
Csn-UF	XbaI	TACGAATCTAGAGATCATTGGCGGAAGTAGAAGTGGAA	151
Csn-UF	BamHI	TTTAGATTGAGTTCATCTGCAGCGGGGATCC GCTTACCGAAAGCCAGACTCAGCAA	152
Csn-UR	BamHI	TTGCTGAGTCTGGCTTCGGTAAGCGGATCC CCGCTGCAGATGAACATCAATCTAAA	153
Csn-DF	BamHI	CGTTAATGCCCATGACAGCCATGAGGATCC GCCAACATGCCCTAGCCCCCTCAC	154
Csn-DR	BamHI	GTGAGAGGGGCTAAGGCTGATTGGCGGATCC TCATGGCTGTCATGGCGCATTAAACG	155
Csn-DR	Sall	ATACTCGTCGACATACGTTGAATTGCCGAGAACCGC	156
Csn-UF-	NA	CTGGAGTACCTGGATCTGGATCTCC	157
Csn-DR-	NA	GCTCGGCTTGTTCAGCTCATTCC	158
SigB-UF	SacI	CGGTTGAGCTCGCGTCCTGATCTGCAGAACGCTATT	159
SigB-UF	BamHI	CTAAAGATGAAGTCGATCGGCTCATGGATCC GCTTACCGAAAGCCAGACTCAGCAA	160
SigB-UR	BamHI	TTGCTGAGTCTGGCTTCGGTAAGCGGATCC ATGAGCCGATCGACTCATCTTAG	161

- 82 -

SigB-DF	BamHI	CGTTAATGCCCATGACAGCCATGAGGATCC GAAGATCCCTCGATGGAGTTAATGT	162
SigB-DR	BamHI	ACATTAACTCCATCGAGGGATCTCGGATCC TCATGGCTGTCATGGCGCATTAACG	163
SigB-DR	Sall	GCTTCGGTCACTTGCCGTCTGGATATCGCTCTCG	164
SigB-UF-	Sacl	GTCAAAGAGCTCTATGACAGCCTCCTCAAATTGCAGG	165
SigB-DR-	Sall	TTCATGTCGACGCTGTGCAAAACCAGCCGGCAGCGCC	166
SpolISA-UF	EcoRI	ACATTGAAATTCAAGCAGGTCAATCAGCTCGCTGACGC	167
SpolISA-UF	BamHI	CCAGCACTGCCCTCCCTCACCCGAAGGATCC GCTTACCGAAAGCCAGACTCAGCAA	168
SpolISA-UR	BamHI	TTGCTGAGTCTGGCTTTCGGTAAGCGGATCC TTCGGGTGAGGGAGCGCAGTGCTGG	169
SpolISA-DF	BamHI	CGTTAATGCCCATGACAGCCATGAGGATCC TCGAGAGATCCGGATGGTTTCCTG	170
SpolISA-DR	BamHI	CAGGAAAACCATCCGGATCTCGAGGATCC TCATGGCTGTCATGGCGCATTAACG	171
SpolISA-DR	HindIII	AGTCAT AAGCTTCTGGCGTTGATTCATCAACGGG	172
SpolISA-UF-	NA	CAGCGCGACTTGTAAAGGGACAATA	173
SpolISA-DR-	NA	GGCTGCTGTGATGAACCTTGTGGA	174

*All deletion constructs include the phleo^R marker

The fragments listed in Tables 3 and 4 were size-verified by gel electrophoresis as described above. If correct, 1 µL each of the upstream, downstream, and antimicrobial resistance marker fragments were placed in a single reaction tube with the DeletionX-UF and DeletionX-DR primers or nested primers where listed. Nested primers are 25 base pairs of DNA homologous to an internal portion of the upstream or downstream fragment, usually about 100 base pairs from the outside end of the fragment (See, Figure 2). The use of nested primers frequently enhances the success of fusion. The PCR reaction components were similar to those described above, except 82 µL of water was used to compensate for additional template volume. The PCR reaction conditions were similar to those described above, except the 72°C extension was lengthened to 3 minutes. During extension, the antimicrobial resistance gene was fused in between the upstream and downstream pieces. This fusion fragment can be directly transformed into *Bacillus* without any purification steps or with a simple Qiagen Qiaquick PRC purification done according to manufacturer's instructions.

EXAMPLE 3**Creation of DNA Constructs Using Ligation of PCR Fragments and Direct Transformation of *Bacillus subtilis* to Bypass the *E. coli* Cloning Step**

5

In this Example, a method ("Method 3") for creating DNA constructs using ligation of PCR fragments and direct transformation of *Bacillus* are described. By way of example, modification of *prpC*, *sigD* and *tdh/kbl* are provided to demonstrate the method of ligation. Indeed, *sigD* and *tdh/kbl* were constructed by one method and *prpC* by an alternate
10 method.

10

A. Tdh/Kbl and SigD

The upstream and downstream fragments adjacent to the *tdh/kbl* region of the *Bacillus subtilis* chromosome were amplified by PCR similar to as described in Method 1, except that the inside primer of the flanking DNA was designed to contain type II s restriction sites. Primers for the loxP-spectinomycin-loxP cassette were designed with the same type II s restriction site as the flanks and complementary overhangs. Unique overhangs for the left flank and the right flank allowed directional ligation of the antimicrobial cassette between the upstream and downstream flanking DNA. All DNA fragments were digested with the appropriate restriction enzymes, and the fragments were purified with a Qiagen Qiaquick PCR purification kit using the manufacturer's instructions. This purification was followed by desalting in a 1 mL spin column containing BioRad P-6 gel and equilibrated with 2 mM Tris-HCl, pH 7.5. Fragments were concentrated to 124 to 250 ng/ μ L using a Savant Speed Vac SC110 system. Three piece ligations of 0.8 to 1 μ g
20 of each fragment were performed with 12U T4 ligase (Roche) in a 15 to 25 μ L reaction volume at 14 to 16°C for 16 hours. The total yield of the desired ligation product was >100 ng per reaction, as estimated by comparison to a standard DNA ladder on an agarose
25 gel. The ligation mixture was used without purification for transformation reactions.
Primers for this construction are shown in Table 5, below

30

- 84 -

Table 5. Primers for tdh/kbl Deletion

Primer Name	Restriction Enzyme Engineered Into Primer	Primer Sequence	SEQ ID NO:
p70 DR	none	CTCAGTTCATCCATCAAATCACCAAGTCCG	175
P82 DF	BbsI	TACACGTTAGAAGACGGCTAGATGCGTCTGATTGTGACAGAC GGCG	176
p71 UF	none	AACCTTCCAGTCCGGTTACTGTCGC	177
		GTACCATAAGAAGACGGAGCTGCCGTGTCCACTCCGATTAT	178
P83 UR	BbsI	AGCAG	
p98spc F	BbsI	CCTTGCTCTGAAGACGGAGCTGGATCCATAACTCGTATAATG	179
p106 spc R	BbsI	GTACCATAAGAAGACGGCTAGAGGATGCATATGGCGGCCGC	180
p112 UF*	none	CATATGCTCCGGCTTCAAGCAAG (analytical primer)	181
p113 DR*	none	CCTGAGATTGATAAACATGAAGTCCTC (analytical primer)	182

5

*primers for analytical PCR

The construct for the sigD deletion closely followed construction of tdh/kbl. The primers used for the sigD construction are provided in Table 6.

10

Table 6. Primers for sigD Construction

Primer Name	Restriction Enzyme Engineered Into Primer	Primer Sequence	SEQ ID NO:
SigD UF	none	ATATTGAAGTCGGCTGGATTGTGG	183
SigD UR	BgIII	GCGGCAGATCTCGGCGCATTAAGTCGTCA	184
SigD DF	EcoRI	GCGGCGAATTCTCTGCTGGAAAAAGTGATACA	185
SigD DR	none	TTCGCTGGATAACAAACAT	186
Loxspc UF	BgIII	GCGGCAGATCTTAAGCTGGATCCATAACTTCG	187
Loxspc DR	EcoRI	GCGGCGAATTCATATGGCGGCCGCATAACTTC	188
SigD UO	none	CAATTACGCGGGGTGGTG	189
SigD DO	none	GAATAGGTTACCGCAGTTGTTG	190
Spc UR	none	CTCCTGATCCAAACATGTAAG	191
Spc DF	none	AACCCTTGATATGTCTAG	192

B. PrpC

An additional example of creating a DNA molecule by ligation of PCR amplified DNA fragments for direct transformation of *Bacillus* involved a partial in-frame deletion of the gene *prpC*. A 3953 bp fragment of *Bacillus subtilis* chromosomal DNA containing the *prpC* gene was amplified by PCR using primers p95 and p96. The fragment was cleaved at unique restriction sites *PflMI* and *BstXI*. This yielded three fragments, an upstream, a downstream, and a central fragment. The latter is the fragment deleted and consists of 170 bp located internal to the *prpC* gene. The digestion mixture was purified with a Qiagen Qiaquick PCR purification kit, followed by desalting in a 1 mL spin column containing BioRad P-6 gel and equilibrated with 2 mM Tris-HCl, pH 7.5. In a second PCR reaction, the antimicrobial cassette, loxP-spectinomycin-loxP, was amplified with the primer containing a *BstXI* site and the downstream primer containing a *PflMI* site both with cleavage sites complementary to the sites in the genomic DNA fragment. The fragment was digested with *PflMI* and *BstXI* and purified as described for the chromosomal fragment above. A three piece ligation of the upstream, antimicrobial cassette, and the downstream fragments was carried out as for *tdh/kbl*, described above. The yield of desired ligation product was similar and the ligation product was used without further treatment for the transformation of *xyl/RcomK* competent *Bacillus subtilis*, as described in greater detail below.

Table 7. Primers for prpC Deletion

Primer Name	Restriction Enzyme Engineered Into Primer	Primer Sequence	SEQ ID NO:
p95 DF	none	GCGCCCTTGATCCTAACAGTCAGATGAAAC	193
p96 UR	none	CGGGTCCGATACTGACTGTAAGTTGAC	194
p100 spc R	<i>PflMI</i>	GTACCATAACCATGCCTGGTAGGATGCATATGGCGGCCGC	195
p101 spc F	<i>BstXI</i>	CCTTGTCTTCCATCTTGCTGGAGCTGGATCCATAACTCGTATAATG	196
p114 anal.	none	GAGAGCAAGGACATGACATTGACGC	197
p115 anal.*	none	GATCTCACCCCTTCAACTTGAAAG	198

- 86 -

C. PckA Deletion

In addition to the above deletions, pckA was also modified. The PCR primers pckA UF, pckA-2Urfus, spc ffus, spc rfus, pckA Dffus and pckA DR, were used for PCR and 5 PCR fusion reactions using the chromosomal DNA of a *Bacillus subtilis* 1168 derivative and pDG1726 (See, Guerout-Fleury et al., Gene 167(1-2):335-6 [1995]) as template. The primers are shown in Table 8. The method used in constructing these deletion mutants was the same as Method 1, described above.

10

Table 8. Primers Used for PckA Deletion

Primer Name	Restriction Enzyme Engineered Into Primer	Primer Sequence	Seq ID NO:
pckA UF	none	TTTGCTTCCTCCTGCACAAGGCCTC	199
pckA-2URfus	none	CGTTATTGTGTGCA TTC CATTGT	200
spc ffus	none	CAATGGAAATGCACACACAATAACGTGACTGGCAA GAGA	201
pckA Dffus	none	GTAATGGCCCTCTCGTATAAAAAAC	202
spc rfus	none	GT TTTTT TACGAGAGGGCCATTACCAATTAGAAT GAATATTCCC	203
pckA DR	none	GACCAAAATGTTCGATT CAGC ATTCCCT	204

15

D. Xylose-Induced Competence Host Cell Transformation with Ligated DNA.

Cells of a host strain *Bacillus subtilis* with partial genotype xylRcomK, were rendered 20 competent by growth for 2 hours in Luria-Bertani medium containing 1% xylose, as described in U.S. Patent Appln. Ser. No. 09/927,161, filed August 10, 2001, herein incorporated by reference, to an OD₅₅₀ of 1. This culture was seeded from a 6 hour culture. All cultures were grown at 37°C, with shaking at 300 rpm. Aliquots of 0.3 mL of were frozen 25 as 1:1 mixtures of culture and 30% glycerol in round bottom 2 mL tubes and stored in liquid nitrogen for future use.

For transformation, frozen competent cells were thawed at 37 °C and immediately after thawing was completed, DNA from ligation reaction mixtures was added at a level of 5 to 15 µL per tube. Tubes were then shaken at 1400 rpm (Tekmar VXR S-10) for 60 min at 37 °C. The transformation mixture was plated without dilution in 100 uL aliquots on 30 8 cm LA plates containing 100 ppm of spectinomycin. After growth over night,

- 87 -

transformants were picked into Luria-Bertani (100 ppm spectinomycin) and grown at 37 °C for genomic DNA isolation performed as known in the art (See e.g., Harwood and Cuttings, Molecular Biological Methods for *Bacillus*, John Wiley and Son, New York, N.Y. [1990], at p. 23). Typically 400 to 1400 transformants were obtained from 100 uL transformation mix, when 5 uL of ligation reaction mix was used in the transformation.

When the antimicrobial marker was located between two *loxP* sites in the incoming DNA, the marker could be removed by transforming the strain with a plasmid containing the *cre* gene capable of expressing the Cre protein. Cells were transformed with pCRM-TS-pleo (See below) cultured at 37 °C to 42 °C, plated onto LA and after colonies formed patched onto LA containing 100 ppm spectinomycin. Patches which did not grow after overnight incubation were deemed to have lost the antimicrobial marker. Loss of marker was verified by PCR assay with primers appropriate for the given gene.

pCRM-TS-pleo has the following sequence (SEQ ID NO:205):

```

15 GGGGATCTCTGCAGTGAGATCTGGTAATGACTCTAGCTTGGCATCAAATAAACGAAAG
   GCTCAGTCGAAAGACTGGGCCTTCGTTTATCTGTTGTTGCGGTGAACGCTCTCGAGTA
   GGACAAATCCGCCCTAGCTAAGCAGAAGGCCATCTGACGGATGGCCTTTGCGTTCT
   ACAAAACTTGTAACTCTAGAGCTGCCTGCCGCTTCGGTGAAGATCTTCCCAGTATTGATT
   AATTAAATTCAAGACGCTCGGTTGCCGCCGGCGTTTTATGCAGCAATGGCAAGAACGTTGC
   10 TCTAGAATAATTCTACACAGCCCCAGTCAGACTATTGGCACTGAAATTATGGGTGAAGTGGTC
   AAGACCTCACTAGGCACCTAAAAATAGCGCACCCCTGAAGAAGATTATTGAGGTAGCCCTT
   GCCTACCTAGCTCCAAGAAAGATATCTAACAGCACAAGAGCGGAAAGATGTTTGTCTACA
   TCCAGAACACCTCTGCTAAAATTCTGAAAAAATTGCAAAAAGTTGTTGACTTATCTACAAG
   GTGTGGCATAATGTGAGGATTGTGAGCGGATAACAATTAAAGCTTAGGAGGGAGTGTAAATG
   25 TCCAATTACTGACCGTACACCAAAATTGCGCTGCATTACCGGTCATGCAACGAGTGTGAG
   GTTCGCAAGAACCTGATGGACATGTTCAAGGGATGCCAGGGCTTCTGAGCATACCTGAAA
   ATGCTTCTGTCGTTGCCGGCTGGCGGATGGTCAAGTTGAATAACCGGAAATGGTT
   CCCGCAGAACCTGAAGATGTCGCGATTATCTTCTATATCTTCAGGGCGCGGGCTGGCAGTA
   AAAACTATCCAGAACATTGGGCCAGCTAAACATGCTTCATCGTCGGTCCGGGCTGCCACGA
   30 CCAAGTGACAGCAATGCTGTTCACTGGTTATGCCGGGATCCGAAAAGAAAAGCTTGTGATGCC
   GGTGAACGTGCAAAACAGGCTTAGCGTTGAACGCACTGATTCGACCAGGTTGTTCACTC
   ATGGAAAATAGCGATCGCTGCCAGGATATACGTAATCTGGCATTCTGGGATTGCTTATAACA
   CCCTGTTACGTATGCCGAAATTGCCAGGATCAGGGTTAAAGATATCTACGTACTGACGGTG
   GGAGAATGTTAATCCATATTGGCAGAACGAAAACGCTGGTTAGCACCGCAGGTGTAGAGAAAG
   35 GCACTTAGCCTGGGGTAACTAAACTGGTCAGCGATGGATTCCGCTCTGGTGTAGCTGAT
   GATCCGAATAACTACCTGTTTGCCGGGTCAGAAAAAAATGGTGTGCGCGCCATCTGCCACC
   AGCCAGCTATCAACTCGCCCTGGAGGGATTGGTGAAGCAACTCATGATTGATTACGGC
   GCTAAGGATGACTCTGGTCAGAGATACCTGGCTGGTCTGGACACAGTGCCTGGCTGGAGC
   CGCGCAGAGATATGCCCGCTGGAGTTCAATACCGGAGATCATGCAAGCTGGTGGCTGGA
   40 CCAATGTAATATTGTCATGAACATATCCGTAACCTGGATAGTGAACACAGGGCAATGGTC
   GCCTGCTGGAAGATGGCGATTAGGAGCTCGCATCACACGCAAAAGGAAATTGGAATAATGC
   GAAATTGAGATGTTAATTAAAGACCTTTGAGGTCTTTCTTAGATTTGGGTTATTTA
   GGGGAGAAAACATAGGGGGTACTACGACCTCCCCCTAGGTGTCCATTGTCCATTGTCAA
   ACAAAATAATAATTGGTTTAATGTTAAAGGTTTTATGTTAAAGTGAACAAAAACAA
   45 GATGTTGGGAGGTACAGTGATAGTTGAGATAGAAAAGAGAAAAAGTTGCTGTTACTTAA
   AGACTTACAACAGAAGAAAATGAGATATTAATAGAATCAAAGAAAATATAATTAGCAAATC
   AGATGCAACCGGTATTCTAATAAAAAAATGCAAAGGAGGAATACGGTGCACTTAAACAAAA
   AAAGATAGACAGCACTGGCATGCTGCCTATCTATGACTAAATTGTTAAGTGTATTAGCACCG
   TTATTATCATGAGCGAAAATGTAATAAAAGAAACTGAAAACAAGAAAATTCAAGAGGACGT

```

- 88 -

AATTGGACATTTGTTTATATCCAGAACATCAGCAAAAGCCGAGTGGTTAGAGTATTTAAAAGAGT
 TACACATTCAATTGTAGTGTCTCCATTACATGATAGGGACTGTGATACAGAAGGTAGGATGAA
 AAAAGAGCATTATCATATTCTAGTGATGTAGGGTAATAAATCTTATGAACAGATAAAAATAA
 5 TATATGCTTCACATGGACGATCTTAATAAATTTAAATATCAAAAAGAAGATATGATAGTTATGG
 CGGTGTAGATGTTGATGAATTAAAGAAAACAACAACAGATAGATATAAATTAATTAAAGAAA
 TGATTGAGTTATTGATGAACAAGGAATCGTAGAATTAAAGAGTTAAGGATTATGGATTATGCAATGAAG
 TTTAAATTTGATGATTGGTCCCCTTTATGTGATAACTCGGGTATGTTATTCAAGAATATAAT
 10 AAAATCAAATCGGTATAAATCTGACCGATAGATTTGAATTAGGTGTACAAGACACTTTT
 TCGCACCAAGCGAAAACGGTTAACGCCGACTGGAGCTCCTGCACGGATGGTGGCGCTGGAT
 GGTAAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTCGCTCCACAAGGTAAACAGTTGAT
 TGAACACTGCCTGAACCTACCGCAGCCGGAGAGCGCCGGCAACTCTGGCTCACAGTACGCGTAG
 TCGAACCGAACCGCACCGCATGGTCAAAGCCGGCACATCAGCGCTGGCAGCAGTGGCG
 TCTGGCGAAAACCTCAGTGTACGCTCCCCGCCGTCCCACGCCATCCCGCATCTGACCA
 15 CCAGCGAAAATGGATTTCATCGAGCTGGTAATAAGCGTTGGCAATTAAACGCCAGTCAG
 GCTTCTTCACAGATGTGGATTGGCGATAAAAACAACGTGTCAGCAGCCGCTGCGCAGTCAGT
 TCACCCGTGCAACCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGCAATTGACCTAAC
 GCCTGGGTCGAACGCTGGAAAGCGGGCATTACCGAGGCCAAGCAGCGTTGTCAG
 GCACGGCAGATAACACTGCTGATGCCGCTGGTCAAGTACGACCCGTCACCGCTGGCAGCATCAG
 20 GGGAAAACCTTATTATCAGCCGGAAAACCTACCGGATTGATGGTAGTGGTCAACTGGCATTGGCAGT
 ACCGTTGATGTTGAAGTGGCGAGCGATAACCCGATCCGGCGCGATTGGCTGAACGAAACTATCCC
 GCTGGCGCAGGTAGCAGAGCGGGTAAACCTGGCTCGGATTAGGCCGAAAGAAAACATATCCC
 GACCGCCTACTGCCGCTGTTTGACCGCTGGGATCTGCCATTGTCAGACATGTATACCCCG
 TACGTCTCCGAGCGAAAACGGCTGCGCTGGGAGCGCCGAAATGGCCACA
 25 CCAGTGGCGCGCGACTTCAACATCAGCCGCTACAGTCAACAGCAACTGATGGAAA
 CCAGCCATCGCCATCTGCTGACCGGAAAGAAGGCCACATGGCTGAATATCAGCGTTCCAT
 ATGGGGATTGGTGGCGACGACTCCTGGAGCCGCTGAGTATCCGGGAAATTCCAGCTGAGCG
 CCGTCGCTACCATTACGAGTGGCTGGTCAAAAATAATAACCGGGCAGGCCATGTC
 TGCCCCTATTGCGTAAGGAAATCCATTATGACTATTCAAGCTAATTCCGGTGGAAACGAG
 30 GTCATCATTCCCGAAAAAACGGTTGCATTAAATCTTACATATGTAATAACTTCAAAGACT
 ACATTGTAAGATTGATGTTGAGTCGGCTGAAAGATGTCAGTACCAATTATTGTTCTGAT
 TGTTCAAGCCATAACACTGTAGGGATAGTGGAAAGAGTGCTTACGATGTCAGACTGCA
 TATTCAAACGGAGGGAGACGATTGATGAAACCGTAACGTTACGATGTCAGAGTATG
 CCGGTGTCTTATCAGACCGTTCCCGCTGGTGAACCAGGCCAGCAGTTCTGCGAAA
 35 CGCGGGAAAAGTGGAAAGCGCGATGGCGGAGCTGAATTACATCTCCAAACCGCGTGGCACAA
 CAACTGGCGGGCAAACAGTCGTTGCTGATTGGCGTGCACCTCCAGTCGCCCCCTGCACGC
 GCCGTGCAAAATTGTCGCGGGGATTAATCTCGCCGATCAACTGGGTGCCAGCGTGGTGG
 TGTCGATGGTAGAACGAAAGCGGGCGTGAAGCCTGTAAGCGGGCGGTGACAATCTCGCG
 CAACCGCTCAGTGGCTGATCATTAACATATCCGCTGGATGACCGAGATGCCATTGCTGTGGAA
 40 GCTGCCTGCACTAATGTTCCGGCTTATTCTGATGTCTGACCGAGACACCCATCAACAGTA
 TTATTCTCCCATGAAGACGGTACGCGACTGGCGTGGAGCATCTGGTCGCAATTGGTCACC
 AGCAAATCGCGCTGTTAGCGGGCCATTAAAGTCTGCTCGCGCGTCTCGTCTGGCTGG
 TGGCATAAATATCTCACTCGCAATCAAATTAGCGGATAGCGGAACGGGAAGGGCAGTGGAGT
 GCCATGTCCGGTTCAACAAACCATGCAAATGCTGAATGAGGGCATCGTCCACTGCGATG
 45 CTGGTTGCAACGATCAGATGGCGTGGCGCAATGCGCGCAGTGCAGCTGGGGCTGC
 GCGTGGTGGCGATATCTCGGTAGTGGGATACGCGATACCGAAGACAGCTCATGTTATATCC
 CGCCGTCAACCACCATCAAACAGGATTTCGCTGCTGGGCAACCCAGCGTGGACCGCTTG
 CTGCAACTCTCTCAGGGCCAGCGGTGAAGGGCAATCAGCTGTTGCCGTCTCGTCTGGTAA
 AAGAAAAACCCCTGGCGCCAATACGCAAACCGCCTCTCCCCGCCGTGGCGATTCA
 50 TAATGCAGCTGGCACGACAGGTTCCCGACTGGAAAGCGGGCAGTGAAGCGCAACGCAATTAA
 TGTGAGTTAGGCATCGCATCCTGCCTCGCGCTTCCGGTATGACGGTAAAAACCTCTGACAC
 ATGCAGCTCCGGAGACGGTACAGCTGTTGAGCGGATGCGGGAGCAGACAAGCCC
 GTCAGGGCGCGTCAGCGGGTGTGGCGGGGTGCGGGCGCAGCCATGACCCAGTCACGTAG
 CGATAGCGGAGGTATACTGGCTTAACATGCGGATCAGAGCAGATTGACTGAGAGTGCAC
 55 CATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCCCTTCC
 GCTTCCCTCGCTCACTGACTCGCTCGCCTGGCTGGCTGGCGAGCGGTATCAGCTCA
 CTCAAAGGGCGTAATACGGTTATCCACAGAACAGGGATAACCGCAGGAAAGAACATGTGAG
 CAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTGTGGCGTTCCATAGG

- 89 -

CTCCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGAC
 AGGACTATAAAGATACCAGGCCTTCCCCCTGGAAGCTCCCTCGCCTCTCCTGTTCCGAC
 CCTGCCGCTTACCGGATACCTGTCGCCCTTCTCCCTCGGAAGCGTGGCGCTTCTCAATG
 CTCACGCTGTAGGTATCTCAGTCGGTAGGTGCTCGCTCCAAGCTGGCTGTGCACG
 5 AACCCCCCGTTCAGCCGACCGCTGCGCTTATCCGTAACACTATCGCTTGAGTCCAACCG
 GTAAGACACGACTTATGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTA
 TGTAGGCGGTGCTACAGAGTTGAAGTGGCTAACACTACGGCTACACTAGAAGGACAGT
 ATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTCGGAAAAGAGTTGGTAGCTTGTAC
 CGGCAAACAAACCACCGCTGGTAGCGGGTTTTTGTGCAAGCAGCAGATTACGCGCAG
 10 AAAAAGGATCTCAAGAAGATCCTTGATCTTCTACGGGCTGACGCTCAGTGGAACGA
 AAACTCACGTTAAGGGATTTGGTATGAGATTATCAAAAAGGATCTTACCTAGATCCTTAA
 ATTAAAAATGAAGTTTAAATCAATCTAAAGTATATGAGTAAACTTGGTCTGACAGTTACCAA
 ATTTGTTAATCATGAGGGCACCTATCTCAGCAGTGTCTATTGTTCATCCATAGTTGCCTGAC
 TGCTTAATCATGAGGGCACCTATCTCAGCAGTGTCTATTGTTCATCCATAGTTGCCTGAC
 TCCCCGTCGTGAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGTGCAATG
 15 ATACCGCGAGACCCACGCTCACCGCTCCAGATTATCAGCAATAAACCAAGCCAGCCGGAAG
 GGCGGAGCGCAGAAGTGGTCTGCAACTTATCCGCTCCATCCAGTCTATTAAATTGTTGCCG
 GGAAGCTAGAGTAAGTAGTTGCCAGTTAATAGTTGCGAACGTTGCGATTGCTACAGG
 CATCGTGGTGTACCGCTCGTCGTTGGTATGGCTTCATTAGCTCCGGTCCCAACGATCAAG
 GCGAGTTACATGATCCCCCATGTTGTGAAAAAGCGGTTAGCTCCTCGGTCTCCGATCGT
 20 TGTCAGAAGTAAGTGGCCGAGTGTATCACTCATGGTTATGGCAGCACTGCATAATTCTT
 ACTGTCATGCCATCCGTAAGATGCTTTCTGACTGGTAGTACTCAACCAAGTCATTGAG
 AATAGTGTATGCGGCACCGAGTTGCTCTGCCCAGTCAACACGGGATAATACGCGCCA
 CATAGCAGAACTTAAAAGTGTCTCATCATTGAAAACGTTCTCGGGGCGAAAACCTCTCAAGG
 25 CTTTACTTCAACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGCACCCAACTGATCTCAGCAT
 ATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGCACCCAACTGATCTCAGCAT
 TATCAGGTTATTGTCATGAGCGGATACATATTGAATGTATTAGAAAAATAACAAATAGG
 TATCAGGTTATTGTCATGAGCGGATACATATTGAATGTATTAGAAAAATAACAAATAGG
 30 AAATGAAAGGGATTATGCGTGAGAATGTTACAGTCTATCCGGCATTGCCAGTCGGGAT
 ATTAAAAAGAGTATAGTTTATTGCGATAAAACTAGGTTCACTTGGTTACCATGAAGATGG
 ATTGCGAGTTCAATGTGTAATGAGGTTGGATTCTATGGGAGGCAAGTGTGAAGGCTG
 GCGCTCTCGTAGTAATGATTACCGGTTGTACAGGTGCGAGTCGTTATTGCTGGTACTGC
 TAGTTGCCGATTGAAGTAGAGGGATTGATGAATTATATCAACATATTAAAGCCTTGGGATT
 35 TTGCAACCAATACATCATTAAAGATCAGTGGGGATGAACGAGACTTGCAGTAATTGATC
 CCGACACAACATTGATTACAATAAAAGCTAAATCTATTAAATCTGTTCTGCAGGAGAGA
 CCG

40 E. Transcriptome DNA Array Methods

In addition to the above methods, transcriptome DNA array methods were used in
 the development of mutants of the present invention. First, target RNA was harvested
 from a *Bacillus* strain by guanidinium acid phenol extraction as known in the art (See e.g.,
 Farrell, RNA Methodologies, (2nd Ed.). Academic Press, San Diego, at pp. 81] and time-
 45 point was reverse-transcribed into biotin-labeled cDNA by a method adopted from
 deSaizieu et al. (deSaizieu et al., J. Bacteriol., 182: 4696-4703 [2000]) and described
 herein. Total RNA (25 mg) was incubated 37°C overnight in a 100-mL reaction: 1x GIBCO
 first-strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂); 10 mM DTT; 40 mM
 random hexamer; 0.3 mM each dCTP, dGTP and dTTP; 0.12 mM dATP; 0.3 mM biotin-
 50 dATP (NEN0; 2500 units SuperScript II reverse-transcriptase (Roche). To remove RNA,

- 90 -

the reaction was brought to 0.25 M NaOH and incubated at 65°C for 30 minutes. The reaction was neutralized with HCl and the nucleic acid precipitated at -20°C in ethanol with 2.5 M ammonium-acetate. The pellet was washed, air-dried, resuspended in water, and quantitated by UV spectroscopy. The reaction yield was approximately 20-25 mg biotin-labeled cDNA.

5 Twelve mg of this cDNA were fragmented in 33 mL 1x One-Phor-All buffer (Amersham-Pharmacia #27-0901-02) with 3.75 milliunits of DNaseI at 37°C for 10 minutes. After heat-killing the DNase, fragmentation was validated by running 2 mg of the fragmented cDNA on a 3% agarose gel. Biotin-containing cDNA routinely ranged in size
10 from 25 to 125 nucleotides. The remaining 10 mg of cDNA were hybridized to an Affymetrix *Bacillus* GeneChip array.

Hybridizations were performed as described in the Affymetrix Expression Analysis Technical Manual (Affymetrix) using reagent suppliers as suggested. Briefly, 10 mg of fragmented biotin-labeled cDNA were added to a 220-mL hybridization cocktail containing:
15 100 mM MES (N-morpholinoethanesulfonic acid), 1M Na⁺, 20 mM EDTA, 0.01% Tween 20; 5 mg/mL total yeast RNA; 0.5 mg/mL BSA; 0.1 mg/mL herring-sperm DNA; 50 pM control oligonucleotide (AFFX-B1). The cocktails were heated to 95°C for 5 minutes, cooled to 40°C for 5 minutes, briefly centrifuged to remove particulates, and 200 mL was injected into each pre-warmed pre-rinsed (1x MES buffer + 5 mg/ml yeast RNA) GeneChip
20 cartridge. The arrays were rotated at 40°C overnight.

The samples were removed and the arrays were filled with non-stringent wash buffer (6x SSPE, 0.01% Tween 20) and washed on the Affymetrix fluidics station with protocol Euk-GE-WS2, using non-stringent and stringent (0.1 M MES, 0.1 M [Na⁺], 0.01% Tween 20) wash buffers. Arrays were stained in three steps: (1) streptavidin; (2) anti-
25 streptavidin antibody tagged with biotin; (3) streptavidin-phycoerythrin conjugate.

The signals in the arrays were detected with the Hewlett-Packard Gene Array Scanner using 570 nm laser light with 3-mm pixel resolution. The signal intensities of the 4351 ORF probe sets were scaled and normalized across all time points comprising a time course experiment. These signals were then compared to deduce the relative expression
30 levels of genes under investigation. The threonine biosynthetic and degradative genes were simultaneous transcribed, indicating inefficient threonine utilization. Deletion of the degradative threonine pathway improved expression of the desired product (See, Figure 7). The present invention provides means to modify pathways with transcription profiles that are similar to threonine biosynthetic and degradative profiles. Thus, the present
35 invention also finds use in the modification of pathways with transcription profiles similar to

- 91 -

threonine in order to optimize *Bacillus* strains. In some preferred embodiments, at least one gene selected from the group consisting of *rocA*, *ycgN*, *ycgM rocF* and *rocD* is deleted or otherwise modified. Using the present invention as described herein resulted in the surprising discovery that the *sigD* regulon was transcribed. Deletion of this gene

5 resulted in better expression of the desired product (See, Figure 7). It was also surprising to find the transcription of *gapB* and *pckA*. Deletion of *pckA* did not result in improvement or detriment. However, the present invention provides means to improve strain protein production through the combination of *pckA* deletion or modification and deletion or modification of *gapB* and/or *fbp*. In addition, during the development of the present

10 invention, it was observed that the tryptophan biosynthetic pathway genes showed unbalanced transcription. Thus, it is contemplated that the present invention will find use in producing strains that exhibit increased transcription of genes such as those selected from the group consisting of *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, and/or *trpF*, such that the improved strains provide improved expression of the desired product, as compared to the

15 parental (i.e., wild-type and/or originating strain). Indeed, it is contemplated that modifications of these genes in any combination will lead to improved expression of the desired product.

F. Fermentations

20 Analysis of the strains produced using the above constructs were conducted following fermentation. Cultures at 14 L scale were conducted in Biolafitte® fermenters. Media components per 7 liters are listed in Table 9.

Table 9. Media Components per 7L Fermentation

25

NaH ₂ PO ₄ -H ₂ O	0.8%	56g
KH ₂ PO ₄	0.8%	56g
MgSO ₄ -7H ₂ O	0.28%	19.6g
antifoam	0.1%	7g
CaCl ₂ -2H ₂ O	0.01%	0.7g
ferrous sulfate-7H ₂ O	0.03%	2.1g
MnCl ₂ -4H ₂ O	0.02%	1.4g
trace metals 100 x stock*	1%	70g
H ₂ SO ₄	0.16%	11.2g

- 92 -

60% glucose	1.29%	90
-------------	-------	----

*See, Harwood and Cutting, *supra*, at p. 549

The tanks were stirred at 750 rpm and airflow was adjusted to 11 Liters per minute, the temperature was 37°C, and the pH was maintained at 6.8 using NH₄OH. A 60%
5 glucose solution was fed starting at about 14 hours in a linear ramp from 0.5 to 2.1 grams per minute to the end of the fermentation. Off-gasses were monitored by mass spectrometry. Carbon balance and efficiency were calculated from glucose fed, yield of protein product, cell mass yield, other carbon in broth, and CO₂ evolved. A mutant strain
10 was compared to parent strain to judge improvements. Although this mutant *pckA* strain did not show improvement under these conditions, it is contemplated that improvements will be produced under modified culture conditions (*i.e.*, as known to those in the art), and/or incorporation of additional genes. In some preferred embodiments, these additional genes are selected from the group consisting of *gapB*, *alsD*, and/or *fbp*

15

EXAMPLE 4

Host Cell Transformation To Obtain An Altered *Bacillus* Strain

Once the DNA construct was created by Method 1 or 2 as described above, it was
20 transformed into a suitable *Bacillus subtilis* lab strain (e.g., BG2036 or BG2097; any competent *Bacillus* immediate host cell may be used in the methods of the present invention). The cells were plated on a selective media of 0.5 ppm phleomycin or 100 ppm spectinomycin as appropriate (Ferrari and Miller, *Bacillus Expression: A Gram-Positive Model in Gene Expression Systems: Using Nature for the Art of Expression*, pgs 65-94 [1999]). The laboratory strains were used as a source of chromosomal DNA carrying the deletion that was transformed into a *Bacillus subtilis* production host strain twice or
25 BG3594 and then MDT 98-113 once. Transformants were streaked to isolate a single colony, picked and grown overnight in 5 mL of LB plus the appropriate antimicrobial. Chromosomal DNA was isolated as known in the art (See e.g., Hardwood *et al.*, *supra*).

30 The presence of the integrated DNA construct was confirmed by three PCR reactions, with components and conditions as described above. For example, two reactions were designed to amplify a region from outside the deletion cassette into the antimicrobial gene in one case (primers 1 and 11) and through the entire insert in another (primers 1 and 12). A third check amplified a region from outside the deletion cassette
35 into the deleted region (primers 1 and 4). Figure 4 shows that a correct clone showed a

- 93 -

band in the first two cases but not the third. Wild-type *Bacillus subtilis* chromosomal DNA was used as a negative control in all reactions, and should only amplify a band with the third primer set.

5 **EXAMPLE 5**

Shake Flask Assays – Measurement of Protease Activity.

Once the DNA construct was stably integrated into a competent *Bacillus subtilis* strain, the subtilisin activity was measured by shake flask assays and the activity was compared to wild type levels. Assays were performed in 250 ml baffled flasks containing 10 50 mL of growth media suitable for subtilisin production as known in the art (See, Christianson *et al.*, Anal. Biochem., 223:119–129 [1994]; and Hsia *et al.*, Anal. Biochem. 242:221 – 227 [1996]). The media were inoculated with 50 µL of an 8 hour 5mL culture and grown for 40 hours at 37°C with shaking at 250 RPM. Then, 1 mL samples were taken at 17, 24 and 40 hours for protease activity assays. Protease activity was measured 15 at 405 nM using the Monarch Automatic Analyser. Samples in duplicate were diluted 1:11 (3.131 g/L) in buffer. As a control to ensure correct machine calibration one sample was diluted 1:6 (5.585 g/L), 1:12 (2.793 g/L and 1:18 (1.862 g/L). Figure 7 illustrates the protease activity in various altered *Bacillus subtilis* clones. Figure 8 provides a graph showing improved protease secretion as measured from shake flask cultures in *Bacillus* 20 *subtilis* wild-type strain (unaltered) and corresponding altered deletion strains (-*sbo*) and (-*sly*). Protease activity (g/L) was measured after 17, 24 and 40 hours.

Cell density was also determined using spectrophotometric measurement at an OD of 600. No significant differences were observed for the samples at the measured time (data not shown).

25

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention 30 as should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the art and/or related fields are intended to be within the scope of the present invention.

CLAIMS

1. A method for enhancing expression of a protein of interest from *Bacillus* comprising:
 - a) obtaining an altered *Bacillus* strain capable of producing a protein of interest, wherein said altered *Bacillus* strain has at least one inactivated chromosomal gene selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spoIIASA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*; and
 - b) growing said altered *Bacillus* strain under conditions such that said protein of interest is expressed by said altered *Bacillus* strain, wherein said expression of said protein of interest is enhanced compared to the expression of said protein of interest in an unaltered *Bacillus* host strain.
2. The method of Claim 1, wherein said protein of interest is selected from the group consisting of homologous proteins and heterologous proteins.
3. The method of Claim 1, wherein said protein of interest is an enzyme selected from the group consisting of proteases, cellulases, amylases, carbohydrases, lipases, isomerases, transferases, kinases, and phosphatases.
4. The method of Claim 3, wherein said protein of interest is a protease.
5. The method of Claim 1, wherein said altered *Bacillus* strain is obtained by deleting one or more chromosomal genes selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spoIIASA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*.
6. An altered *Bacillus* strain obtained using the method of Claim 1.
7. An altered *Bacillus* strain comprising a chromosomal deletion of one or more genes selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spoIIASA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*.

8. The altered *Bacillus* strain of Claim 7, wherein said altered strain is a *B. subtilis* strain.

9. The altered *Bacillus* strain of Claim 7, wherein said altered *Bacillus* strain is a protease producing strain.

10. The altered *Bacillus* strain of Claim 9, wherein said protease is a subtilisin.

11. The altered *Bacillus* strain of Claim 11, wherein said subtilisin is selected from the group consisting of subtilisin 168, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin 147, subtilisin 309 and variants thereof.

12. The altered *Bacillus* strain of Claim 7, wherein said altered *Bacillus* strain further comprises a mutation in a gene selected from the group consisting of *degU*, *degQ*, *degS*, *scoC4*, *spolIE*, and *oppA*.

13. The altered *Bacillus* strain of Claim 7, wherein said altered *Bacillus* strain further comprises a heterologous protein of interest.

14. A DNA construct comprising at least one gene selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*, gene fragments thereof, and homologous sequences thereto.

15. The DNA construct of Claim 14, wherein said at least one gene comprises at least one nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 37, SEQ ID NO: 25, SEQ ID NO: 21, SEQ ID NO: 50, SEQ ID NO: 29, SEQ ID NO: 23, SEQ ID NO: 27, SEQ ID NO: 19, SEQ ID NO: 31, SEQ ID NO: 48, SEQ ID NO: 46, SEQ ID NO: 35, and SEQ ID NO: 33.

16. The DNA construct of Claim 14, wherein said construct further comprises a polynucleotide sequence encoding a protein of interest.

- 96 -

17. A plasmid comprising the DNA construct of Claim 16.
18. A host cell comprising the plasmid of Claim 17.
19. The host cell of Claim 18, wherein said host cell is selected from the group consisting of *Bacillus* cells and *E. coli* cells.
20. The host cell of Claim 19, wherein said host cell is *B. subtilis*.
21. The host cell of Claim 18, wherein said DNA construct has been integrated into the chromosome of said host cell.
22. The DNA construct of Claim 14, wherein said at least one gene encodes at least one amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 38, SEQ ID NO: 26, SEQ ID NO: 22, SEQ ID NO: 57, SEQ ID NO: 30, SEQ ID NO: 24, SEQ ID NO: 28, SEQ ID NO: 20, SEQ ID NO: 32, SEQ ID NO: 55, SEQ ID NO: 53, SEQ ID NO: 36, and SEQ ID NO: 34.
23. The DNA construct of Claim 14, further comprising a selective marker, wherein the selective marker is flanked on each side by a fragment of said gene or homologous gene sequence thereto.
24. A DNA construct comprising an incoming sequence, wherein said incoming sequence comprises a nucleic acid encoding a protein of interest, and a selective marker flanked on each side with a homology box, wherein said homology box includes nucleic acid sequences having 80 to 100% sequence identity to the sequence immediately flanking the coding regions of at least one gene selected from the group consisting of *sbo*, *slr*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*.
25. The DNA construct of Claim 24, further comprising at least one nucleic acids which flanks the coding sequence of said gene.

- 97 -

26. A plasmid comprising the DNA construct of Claim 25.
27. A host cell comprising the plasmid of Claim 26.
28. The host cell of Claim 27, wherein said host cell is selected from the group consisting of *Bacillus* cells and *E. coli* cells.
29. The host cell of Claim 28, wherein said host cell is *B. subtilis*.
30. The host cell of Claim 26, wherein said DNA construct has been integrated into the host cell chromosome.
31. The host cell of Claim 30, wherein said selective marker has been excised from said host cell chromosome.
32. A method for obtaining an altered *Bacillus* strain with enhanced protease production comprising:
 - a) transforming a *Bacillus* host cell with the DNA construct of Claim 14, wherein said protein of interest in said DNA construct is a protease, and wherein said DNA construct is integrated into the chromosome of the *Bacillus* host cell under conditions such that said at least one gene is inactivated to produce an altered *Bacillus* strain; and
 - b) growing said altered *Bacillus* strain under conditions such that enhanced protease production is obtained.
33. The method of Claim 32, further comprising recovering said protease.
34. The method of Claim 32, wherein said at least one inactivated gene is deleted from the chromosome of said altered *Bacillus* strain.
35. An altered *Bacillus* strain produced using the method of Claim 32.
36. The method of Claim 33, wherein said *Bacillus* host strain is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. brevis*, *B.*

- 98 -

stearothermophilus, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. laetus*, *B. clausii*, *B. megaterium*, and *B. thuringiensis*.

37. The method of Claim 36, wherein said *Bacillus* host cell is *B. subtilis*.

38. A method for enhancing expression of a protease in an altered *Bacillus* comprising:

- a) transforming a *Bacillus* host cell with the DNA construct of Claim 24;
- b) allowing homologous recombination of said DNA construct and a region of the chromosome of said *Bacillus* host cell, wherein at least one gene of said chromosome of said *Bacillus* host cell is inactivated, to produce an altered *Bacillus* strain; and
- c) growing said altered *Bacillus* strain under conditions suitable for the expression of said protease, wherein the production of said protease is greater in the altered *Bacillus subtilis* strain compared to said *Bacillus subtilis* host prior to transformation in step a).

39. The method of Claim 38, wherein said protease is subtilisin.

40. The method of Claim 38, wherein said protease is a recombinant protease.

41. The method of Claim 38, wherein said inactivation is by deletion of at least one of said genes.

42. The method of Claim 38, wherein said inactivation is by insertional inactivation of said at least one of said genes.

43. The altered *Bacillus* strain obtained using the method of Claim 38.

44. The altered *Bacillus* strain of Claim 38, wherein said altered *Bacillus* strain comprises at least one inactivated gene selected from the group consisting of *sbo*, *sir*, *ybcO*, *csn*, *spolISA*, *sigB*, *phrC*, *rapA*, *CssS*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE*, *trpF*, *tdh/kbl*, *alsD*, *sigD*, *prpC*, *gapB*, *pckA*, *fbp*, *rocA*, *ycgN*, *ycgM*, *rocF*, and *rocD*.

- 99 -

45. The altered *Bacillus* strain of Claim 44, wherein said inactivated gene has been inactivated by deletion.

46. The altered *Bacillus* strain of Claim 44, further comprising at least one mutation in a gene selected from the group consisting of *degU*, *degS*, *degQ*, *scoC4*, *spolE*, and *oppA*.

47. The altered *Bacillus* strain of Claim 46, wherein said mutation is *degU(Hy)32*.

48. The altered *Bacillus* strain of Claim 44, wherein said strain is a recombinant protease producing strain.

49. The altered *Bacillus* strain of Claim 44, wherein said altered *Bacillus* strain is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens* *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. laetus*, *B. clausii*, *B. megaterium*, and *B. thuringiensis*.

50. An altered *Bacillus* strain comprising a deletion of one or more indigenous chromosomal regions or fragments thereof, wherein said indigenous chromosomal region includes about 0.5 to 500 kb, and wherein said altered *Bacillus* strain has an enhanced level of expression of a protein of interest compared to a corresponding unaltered *Bacillus* strain when said altered and unaltered *Bacillus* strains are grown under essentially the same growth conditions.

51. The altered *Bacillus* strain of Claim 50, wherein said altered *Bacillus* strain is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens* *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. laetus*, *B. clausii*, *B. megaterium*, and *B. thuringiensis*.

52. The altered *Bacillus* strain of Claim 51, wherein said altered *Bacillus* strain is selected from the group consisting of *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*.

- 100 -

53. The altered *Bacillus* strain of Claim 52, wherein said altered *Bacillus* strain is a *B. subtilis* strain.

54. The altered *Bacillus* strain of Claim 50, wherein said indigenous chromosomal region is selected from the group consisting of a PBSX region, a skin region, a prophage 7 region, a SP β region, a prophage 1 region, a prophage 2 region, a prophage 4 region, a prophage 3 region, a prophage 4 region, a prophage 5 region, a prophage 6, region, a PPS region, a PKS region, a YVFF-YVEK region, a DHB region and fragments thereof.

55. The altered *Bacillus* strain of Claim 50, wherein two indigenous chromosomal regions or fragments thereof have been deleted.

56. The altered *Bacillus* strain of Claim 50, wherein said protein of interest is a protease.

57. The altered *Bacillus* strain of Claim 56, wherein said protease is a subtilisin.

58. The altered *Bacillus* strain of Claim 57, wherein said subtilisin is selected from the group consisting of subtilisin 168, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin 147 and subtilisin 309 and variants thereof.

59. The altered *Bacillus* strain of Claim 50, wherein said *Bacillus* host is a recombinant strain.

60. The altered *Bacillus* strain of Claim 50, further comprising at least one mutation in a gene selected from the group consisting of *degU*, *degQ*, *degS*, *sco4*, *spolIE* and *oppA*.

61. A protease producing *Bacillus* strain comprising a deletion of an indigenous chromosomal region selected from the group consisting of a PBSX region, a skin region, a prophage 7 region, a SP β region, a prophage 1 region, a prophage 2 region, a prophage 3 region, a prophage 4 region, a prophage 5 region, a prophage 6 region, a PPS region, a PKS region, a YVFF-YVEK region, a DHB region and fragments thereof.

- 101 -

62. The protease producing *Bacillus* strain of Claim 61, wherein said protease is a subtilisin.

63. The protease producing *Bacillus* strain of Claim 62, wherein said *Bacillus* is a *B. subtilis* strain.

64. The protease producing *Bacillus* strain of Claim 61, wherein said protease is a heterologous protease.

65. A method for enhancing the expression of a protein of interest in *Bacillus* comprising:

a) introducing a DNA construct including a selective marker and an inactivating chromosomal segment into a *Bacillus* host strain, wherein said DNA construct is integrated into the chromosome of said *Bacillus* host strain, resulting in the deletion of an indigenous chromosomal region or fragment thereof from said *Bacillus* host cell to produce an altered *Bacillus* strain; and

b) growing said altered *Bacillus* strain under suitable conditions, wherein expression of a protein of interest is greater in the altered *Bacillus* strain compared to the expression of the protein of interest in a *Bacillus* host cell that has not been altered.

66. The method of Claim 65, further comprising recovering said protein of interest.

67. The method of Claim 65, further comprising the step of excising said selective marker from the altered *Bacillus* strain.

68. The method of Claim 65, wherein said indigenous chromosomal region is selected from the group of regions consisting of PBSX, SKIN, prophage 7, SP β , prophage 1, prophage 2, prophage 3, prophage 4, prophage 5, prophage 6, PPS, PKS, YVFF-YVEK, DHB and fragments thereof.

69. The method of Claim 65, wherein said altered *Bacillus* strain comprises deletion of at least two indigenous chromosomal regions.

70. The method of Claim 65, wherein said protein of interest is an enzyme.

- 102 -

71. The method of Claim 65, wherein said *Bacillus* host strain is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. brevis*, *B. stearothermophilus*, *B. clausii*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus* and *B. thuringiensis*.

72. An altered *Bacillus* strain produced using the method of Claim 65.

73. A method for obtaining a protein of interest from a *Bacillus* strain comprising:

- a) transforming a *Bacillus* host cell with a DNA construct comprising a selective marker and an inactivating chromosomal segment, wherein said DNA construct is integrated into the chromosome of the *Bacillus* strain resulting in deletion of an indigenous chromosomal region or fragment thereof, to produce an altered *Bacillus* strain,
- b) culturing said altered *Bacillus* strain under suitable growth conditions to allow the expression of a protein of interest, and
- c) recovering said protein of interest.

74. The method of Claim 73, wherein said protein of interest is an enzyme.

75. The method of Claim 73, wherein said *Bacillus* host comprises a heterologous gene encoding a protein of interest.

76. The method of Claim 73, wherein said *Bacillus* host cell is selected from the group consisting of *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. brevis*, *B. stearothermophilus*, *B. clausii*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus* and *B. thuringiensis*.

77. The method of Claim 73, wherein said indigenous chromosomal region is selected from the group of regions consisting of PBSX, SKIN, prophage 7, SP β , prophage 1, prophage 2, prophage 3, prophage 4, prophage 5, prophage 6, PPS, PKS, YVFF-YVEK, DHB and fragments thereof.

- 103 -

78. The method of Claim 77, wherein said altered *Bacillus* strain further comprises at least one mutation in a gene selected from the group consisting of *degU*, *degQ*, *degS*, *sco4*, *spolIE* and *oppA*.

79. The method of Claim 73, wherein said protein of interest is an enzyme selected from the group consisting of proteases, cellulases, amylases, carbohydrases, lipases, isomerases, transferases, kinases, and phosphatases.

80. The method of Claim 79, wherein said enzyme is a protease.

81. A method for enhancing the expression of a protein of interest in *Bacillus* comprising:

- a) obtaining nucleic acid from at least one *Bacillus* cell;
- b) performing transcriptome DNA array analysis on said nucleic acid from said *Bacillus* cell to identify at least one gene of interest;
- c) modifying said at least one gene of interest to produce a DNA construct;
- d) introducing said DNA construct into a *Bacillus* host cell to produce an altered *Bacillus* strain, wherein said altered *Bacillus* strain is capable of producing a protein of interest, under conditions such that expression of said protein of interest is enhanced as compared to the expression of said protein of interest in a *Bacillus* that has not been altered.

82. The method of Claim 81, wherein said protein of interest is associated with at least one biochemical pathway selected from the group consisting of amino acid biosynthetic pathways and biodegradative pathways.

83. The method of Claim 82, wherein said biodegradative pathway is disabled by transcription of said gene of interest.

84. A method for enhancing the expression of a protein of interest in *Bacillus*, comprising:

- a) obtaining nucleic acid containing at least one gene of interest from at least one *Bacillus* cell;
- b) fragmenting said nucleic acid;

- 104 -

- c) amplifying said fragments to produce a pool of amplified fragments comprising said at least one gene of interest;
- d) ligating said amplified fragments to produce a DNA construct;
- e) directly transforming said DNA construct into a *Bacillus* host cell to produce an altered *Bacillus* strain;
- f) culturing said altered *Bacillus* strain under conditions such that expression of said protein of interest is enhanced as compared to the expression of said protein of interest in a *Bacillus* that has not been altered.

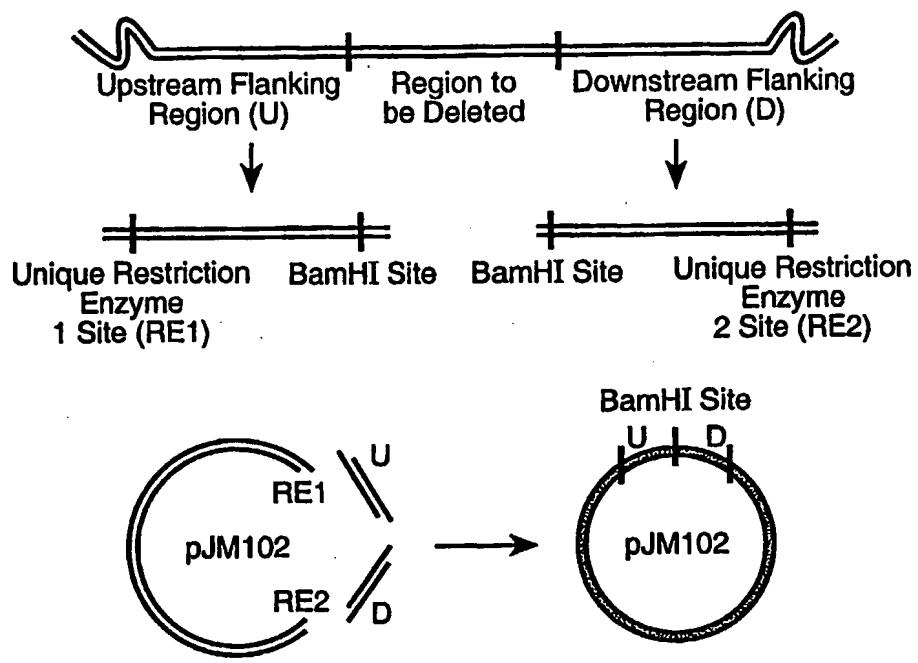
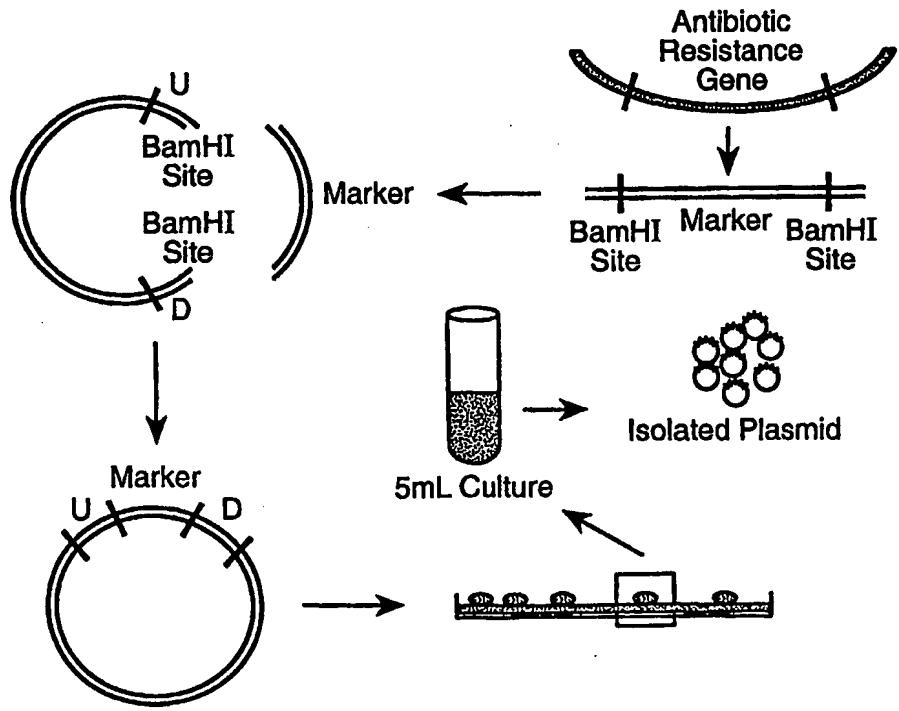
85. The method of Claim 84, wherein said altered *Bacillus* strain comprises modified gene selected from the group consisting of *prpC*, *sigD* and *tdh/kbl*.

86. An isolated nucleic acid comprising the sequence set forth in a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:37, SEQ ID NO:25, SEQ ID NO:21, SEQ ID NO:50, SEQ ID NO:23, SEQ ID NO:27, SEQ ID NO:19, SEQ ID NO:31, SEQ ID NO:48, SEQ ID NO:46, SEQ ID NO:35, and SEQ ID NO:33.

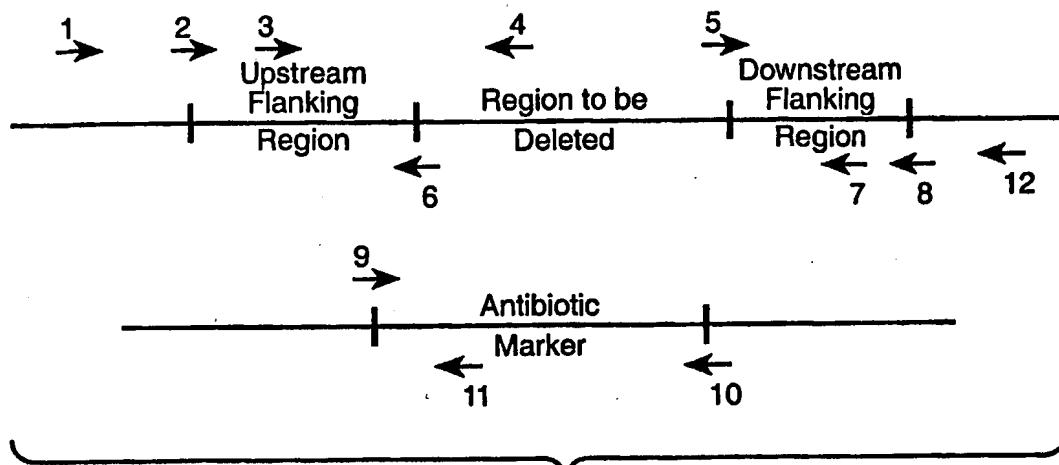
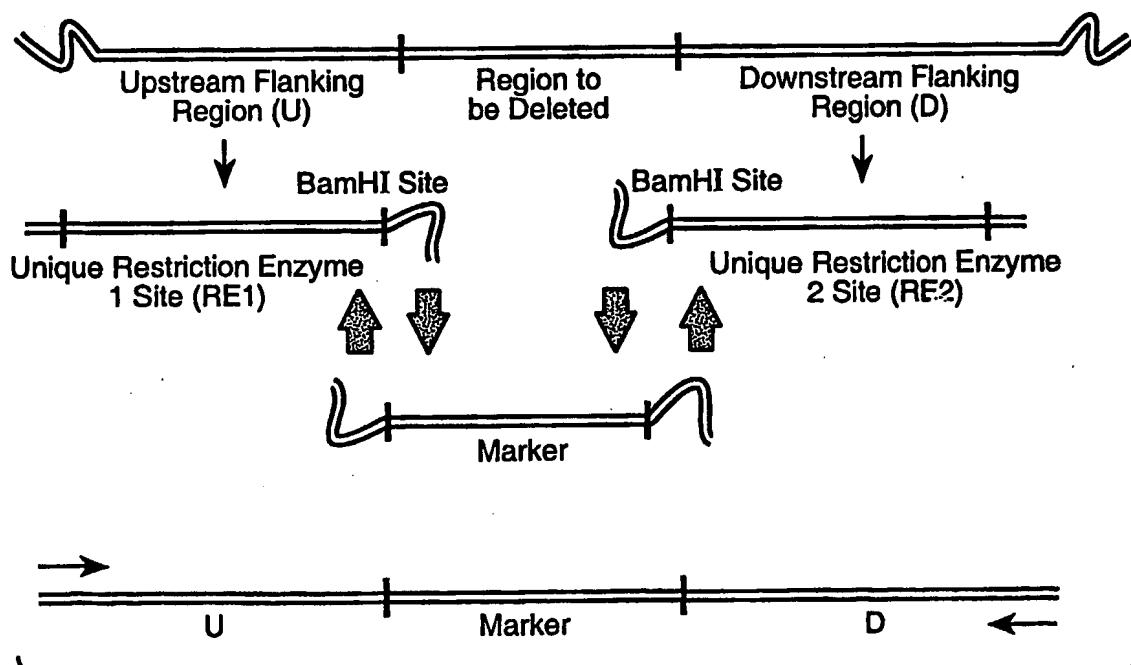
87. An isolated nucleic acid sequence encoding an amino acid, wherein said amino acid is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:38, SEQ ID NO:26, SEQ ID NO:22, SEQ ID NO:57, SEQ ID NO:24, SEQ ID NO:28, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:55, SEQ ID NO:53, SEQ ID NO:36, and SEQ ID NO:34.

88. An isolated amino acid sequence, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:38, SEQ ID NO:26, SEQ ID NO:22, SEQ ID NO:57, SEQ ID NO:24, SEQ ID NO:28, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:55, SEQ ID NO:53, SEQ ID NO:36, and SEQ ID NO:34.

1 / 5

**FIG._ 1A****FIG. 1B**

2 / 5

**FIG._2****FIG._3**

3 / 5

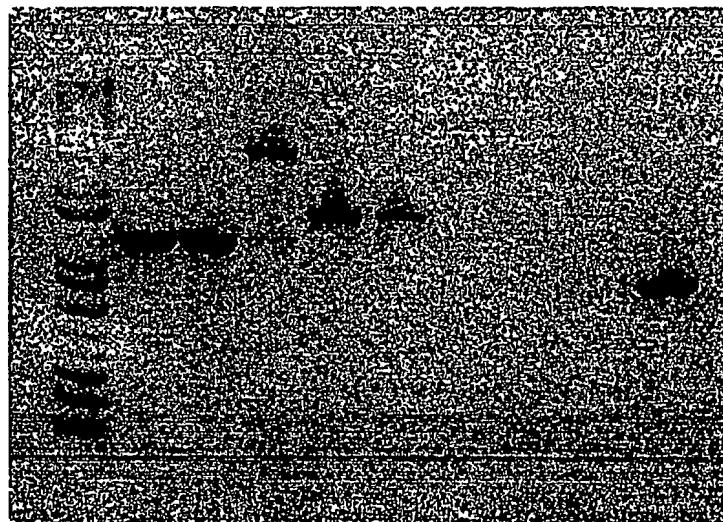


FIG._4

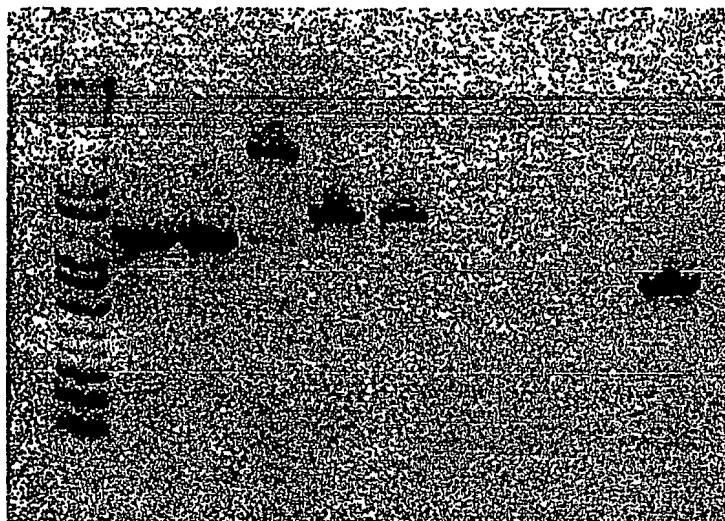


FIG._5

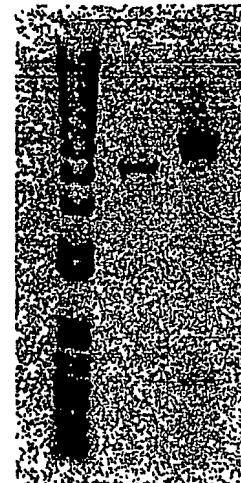
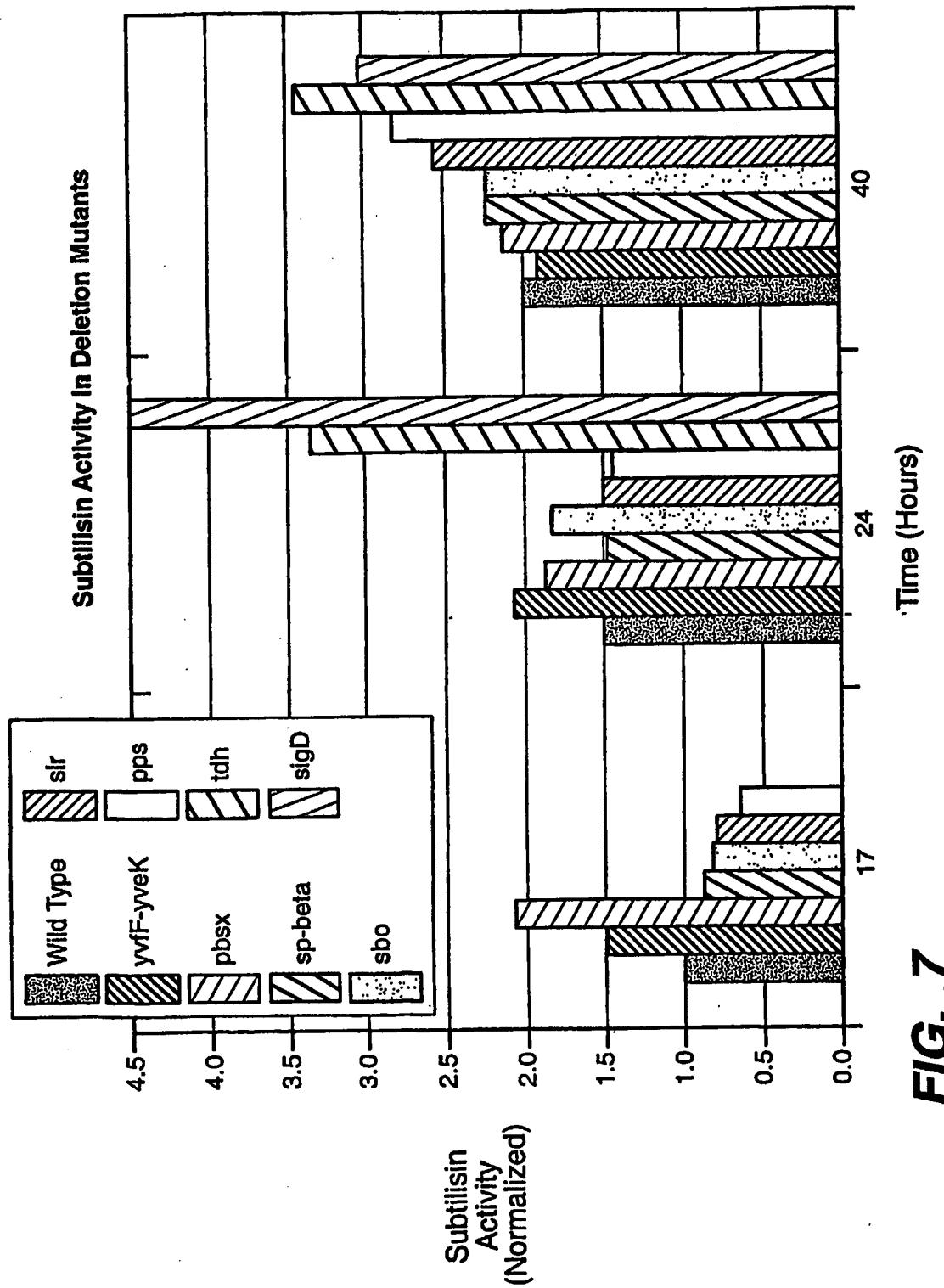


FIG._6

4 / 5

**FIG. 7**

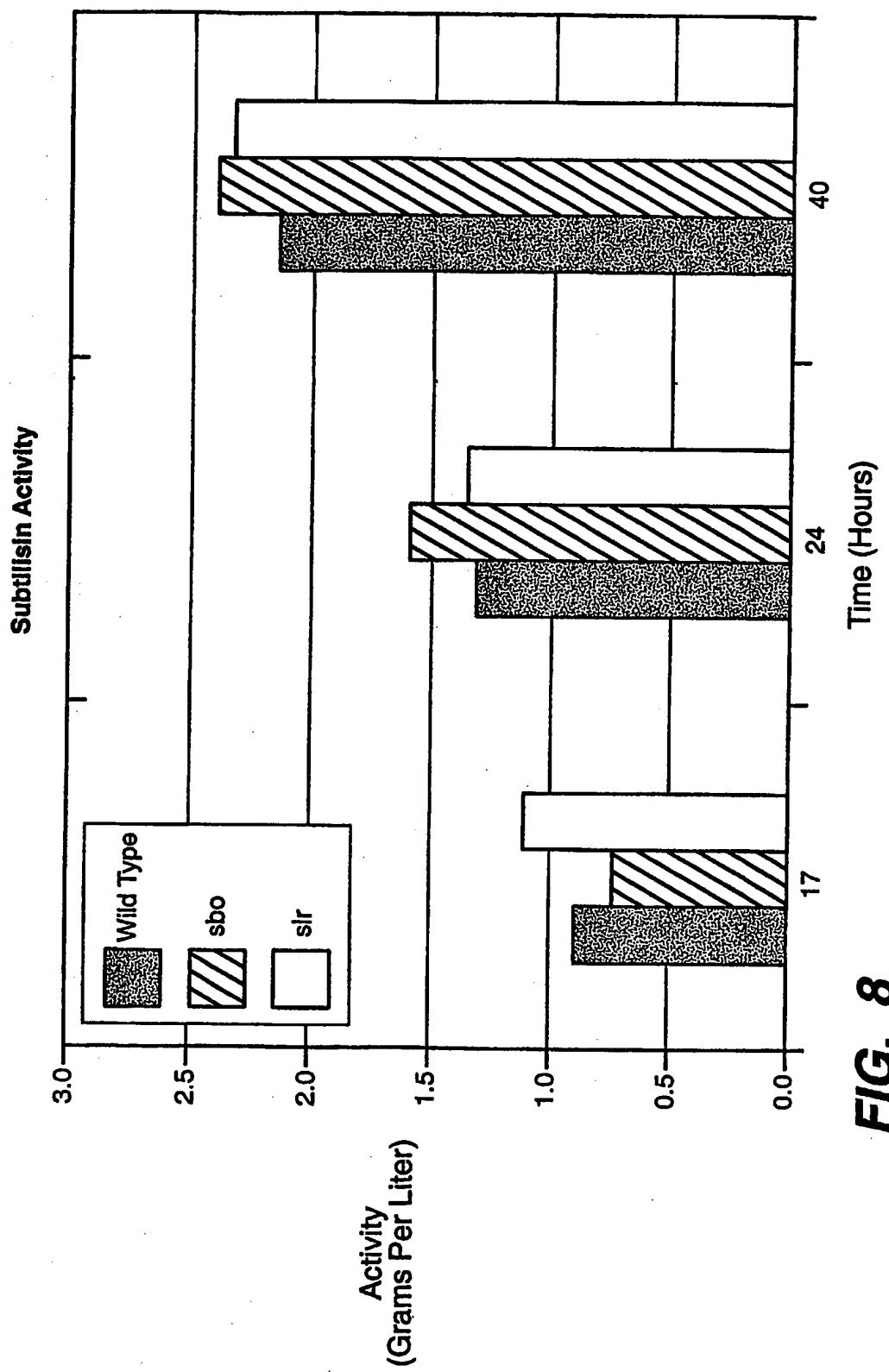


FIG._8

INTERNATIONAL SEARCH REPORT

International application No. PCT/US03/09585

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 21/06; C12N 9/54, 1/20, 15/00, 15/74, 1/00
 US CL : 435/252.31, 252.5, 221, 69.1, 440, 832, 471, 69.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 U.S. : 435/252.31, 252.5, 221, 69.1, 440, 832, 471, 69.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,387,521 A (FERRARI) 07 February 1995, see entire document.	1-13
Y	YANG et al. Alanine-scanning mutagenesis of <i>Bacillus subtilis</i> trp RNA-binding attenuation protein (TRAP) reveals residues involved in tryptophan binding and RNA binding. <i>J. Mol. Biol.</i> 1997, Vol. 270, pages 696-710, see entire document.	1-13
Y, P	US 6,528,255 B1 (ESTELL) 04 March 2003, see entire document.	1-13
Y	US 5,585,253 A (DOI et al.) 17 December 1996, see entire document.	1-13
Y	WO 99/03984 A2 (GENENCOR INTERNATIONAL, INC) 28 January 1999, see entire document.	1-13
A	YAKHNIN et al. Expression of the <i>Bacillus subtilis</i> trpHDCFBA operon is influenced by translational coupling and Rho termination factor. <i>J. Bacteriology</i> . October 2001, Vol. 183, No. 20, pages 5918-5926, see entire document.	1-13
A	EP 0 246 678 A1 (GENENTECH, INC) 25 November 1987, see entire document.	1-13

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"A" document defining the general state of the art which is not considered to be of particular relevance

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"B" earlier application or patent published on or after the international filing date

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"Z"

document member of the same patent family

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search

29 July 2003 (29.07.2003)

Date of mailing of the international search report

22 AUG 2003

Name and mailing address of the ISA/US

Authorized officer

Mail Stop PCT, Attn: ISA/US
 Commissioner for Patents
 P.O. Box 1450
 Alexandria, Virginia 22313-1450

Nina M. Mannfield

Faxsimile No. (703)305-3230

Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

PCT/US03/09585

INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92/19721 A1 (GENENCOR INTERNATIONAL, INC) 12 November 1992, see entire document.	1-13
Y	US 5,310,675 A (ESTELL et al) 10 May 1994, see entire document.	1-13
Y	US 5,264,366 A (FERRARI et al) 23 November 1993, see entire document.	1-13
A,P	US 6,509,185 B1 (VALLE et al) 21 January 2003, see entire document.	1-13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/09585

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-13

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

Group I, claims 1-13, drawn to altered Bacillus strain and method for enhancing expression of protein.

Group II, claims 14-37, drawn to DNA construct.

Group III, claims 38-49, drawn to method for enhancing expression of a protease.

Group IV, claims 50-60, drawn to an altered Bacillus strain.

Group V, claims 61-64, drawn to protease producing Bacillus strain.

Group VI, claims 65-72, drawn to method for expression of a protein of interest.

Group VII, claims 73-80, drawn to method for obtaining a protein of interest from a Bacillus strain.

Group VIII, claims 81-83, drawn to method for enhancing expression of a protein of interest in Bacillus.

Group IX, claims 84-85, drawn to method for enhancing expression of a protein of interest in Bacillus.

Group X, claims 86-87, drawn to a nucleic acid sequence.

Group XI, claim 88, drawn to an amino acid sequence.

The inventions listed as Groups I-XI do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Groups I, II, IV, X and XI are drawn to different products each defining different technical features that lack unity of invention. The technical feature of Group I is not special in view of the teachings of Ferrari (5387521). Ferrari discloses an altered Bacillus strain that is capable of expressing proteins/enzymes. As such the various products and methods lack unity of invention because the technical feature of Group I is not "special" in that it does not define a novel contribution over the prior art. As such, the groups of inventions lack a corresponding "special" technical feature.

Continuation of B. FIELDS SEARCHED Item 3:

WEST, CABA, BIOSIS, MEDLINE, JAPIO, WPIDS, BIOTECHDS, LIFESCI, CAPLUS, AGRICOLA

search terms: inventor names, bacillus strain, alter?, mutant?, mutat?, gene, heterologous protein, express?, inactivat? chromosomal gene, recited genes in claim 1